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VITAMIN B_6 REQUIREMENT OF YOUNG ADULT WOMEN USING ORAL CONTRACEPTIVES ASSESSED BY PYRIDOXAL AND AMINOTRANSFERASE LEVELS IN THE ERYTHROCYTE

by

© TERESA R. BOSSÉ

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Vitamin B_6 requirement of young adult women using oral contraceptives assessed by pyridoxal and aminotransferase levels in the erythrocyte" submitted by Teresa R. Bossé, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

Eight female university students who were using oral contraceptive (OC) preparations of various types and 8 students, similar in age and weight but not using OC served as subjects in a study which investigated the requirement of OC users for vitamin B_6. Both groups of subjects consumed a basal diet consisting of natural foods of known composition and containing 0.36 mg of vitamin B_6. For the first 10 days of the study they were given an additional supplement of 1.70 mg of pyridoxine hydrochloride (PIN-HCl). This period served as an adjustment phase which permitted the determination of base-line values in both OC users and nonusers, and also familiarized the subjects with the metabolic routine.

Only the OC users underwent the remainder of the study. Immediately after the 10 days of adjustment, the supplement of PIN-HCl was removed, resulting in a total daily intake of 0.36 mg of vitamin B_6. This diet was consumed from day 11 to day 42, or a total of 32 days, and was then followed by a repletion phase. Repletion was executed in 3 consecutive steps: 0.96 mg, 1.56 mg and 5.06 mg of vitamin B_6 was consumed for 7, 8 and 6 days respectively. Fasting blood samples were drawn on the mornings of day 2, 11, 18, 25, 32, 39, 43, 50, 59 and 66. Vitamin B_6 nutriture was monitored by analyzing these blood samples for 3 parameters in the erythrocyte: pyridoxal (E-PAL) concentration, enzyme activity and pyridoxal phosphate (PAL-P) stimulation of alanine aminotransferase (E-Ala-AT) and aspartate aminotransferase (E-Asp-AT).

Comparison of mean E-PAL concentration and mean E-Ala-AT activity in the OC users with the nonusers at the end of the adjustment period showed no significant differences between the 2 groups for either parameter. In vitro mean PAL-P percent stimulation of E-Ala-AT in the OC using group

was significantly lower ($P < 0.025$). E-Asp-AT activity in OC users was significantly higher ($P < 0.05$) than that of nonusers. No significant differences were noted between the 2 groups when in vitro PAL-P percent stimulation of E-Asp-AT was done.

Having undergone depletion of vitamin B_6 for 32 days, mean E-PAL concentration of the OC users declined to 49% of the mean undepleted concentration. Repletion with 0.96 mg of vitamin B_6 increased mean E-PAL concentration to 74% of the mean undepleted level. Although repletion with vitamin B_6 at the 1.56 mg/day level returned mean E-PAL concentration to the starting level, only 5 of the 8 subjects had their individual starting values restored. Repletion with vitamin B_6 at the 5.06 mg/day level was effective in restoring E-PAL levels to undepleted values in all subjects.

Mean E-Ala-AT activity dropped to 45% of the mean undepleted activity after the 32 days of depletion. Repletion with 0.96 mg of vitamin B_6 for 7 days and 1.56 mg for 8 days increased mean enzyme activity to 49% and 77% of the mean undepleted activity, respectively. Further repletion at the 5.06 mg/day level for 6 days increased E-Ala-AT activity to 94% of the mean undepleted activity. Even at this highest level of repletion only 4 of the 8 subjects had their individual levels normalized. Although mean in vitro PAL-P stimulation of E-Ala-AT fluctuated somewhat, the general trend was one of increasing percent stimulation as depletion progressed and decreasing percent stimulation with repletion of vitamin B_6.

Basal and percent PAL-P stimulated activity of E-Asp-AT of the OC users during depletion and repletion did not consistently reflect vitamin B_6 intake. The reliability of this parameter for use in de-

termining vitamin B_6 status in OC users was questioned.

The results of this study indicated that at least some OC users had need for vitamin B_6 in amounts that were in excess of recommended allowances for normal women. Comparison of E-PAL and E-Ala-AT in OC users and nonusers while consuming approximately 2 mg of vitamin B_6 showed no significant difference between the 2 groups; thus, evidence of spontaneous vitamin B_6 deficiency in erythrocytes of OC users was not detected. Data from the E-PAL and E-Ala-AT analyses of the OC users after depletion of body stores indicated that the amount of vitamin B_6 required to restore these depleted values to normal was in excess of the amounts of the vitamin recommended as desirable for normal young women, thus suggesting lower initial body stores. A daily intake of approximately 5 mg of vitamin B_6 was effective in restoring completely E-PAL to normal, while the same intake had not quite restored E-Ala-AT activity.

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INTRODUCTION

Women using oral contraceptives containing synthetic estrogen and progestogen combinations have been shown to excrete abnormally high levels of tryptophan metabolites following a load dose of tryptophan. These abnormalities are correctable by large doses of pyridoxine hydrochloride. The above findings suggest a subclinical vitamin B_6 deficiency and thus, an increased requirement for the vitamin by those women using oral contraceptives. Other parameters are known to reflect vitamin B_6 nutritional status, including blood levels of the vitamin, activities of vitamin B_6 dependent aminotransferases, and urinary excretion of vitamin B_6 and 4-pyridoxic acid. These latter parameters have not been used extensively to assess vitamin B_6 status in women using oral contraceptives.

The present study was undertaken to evaluate vitamin B_6 nutriture, using a multiparameter approach, in a group of young women using oral contraceptives and to compare them to a group of young women not using these preparations. The objectives were:

1) to assess the vitamin B_6 nutritional status of young women using oral contraceptives,

2) to determine whether young women using oral contraceptives require more vitamin B_6 than nonusers and

3) to establish what this requirement might be.

Reported here are the results of the pyridoxal concentration, and the activity of alanine aminotransferase and aspartate aminotransferase in the erythrocyte. Other parameters were also studied: excretory levels of tryptophan metabolites, 4-pyridoxic acid and vitamin B_6, but are not reported in this thesis.

REVIEW OF LITERATURE

> "The process of human reproduction, previously essential to the preservation of the species, now poses a severe and very real threat to the continuing of a species". (1)

This statement by A. C. Barnes (1) expresses well man's changing emphasis on human fertility and his realization that population growth cannot continue indefinitely. Man is becoming increasingly aware that the supply of natural resources is not inexhaustible and that limitations exist in the space available to support a population. There is mounting realization by developing nations that fertility control is a vital measure in the aversion of economic and social problems arising from rapid population growth. Well established, government sponsored family planning programs already exist in India, Pakistan, Taiwan, Korea, Singapore and Hong Kong (2). Fertility has declined in these countries.

Family planning in a large, well developed nation such as Canada is still a matter of personal choice as population control per se is not as critical as in a developing nation. Yet the birth rate in Canada has sharply declined from 28.6 per thousand population in 1960 to 17.6 per thousand in 1969 (3). The general fertility rate (birth rate per 1,000 women, 15 to 49 years) has also decreased from a high of 118.0 in 1957 to 71.7 in 1969 (3). These statistics exemplify the increasing, voluntary tendency to limit family size in the Western world.

At the present time the oral contraceptive steroid, or what is commonly called "the pill", is the most widely used method of contraception because of its effectiveness and convenience. The pill includes a wide array of synthetic estrogens and progestogens, taken either in a

combined preparation or in a sequential formulation. The result of either type is multiple interference with several phases of the reproductive cycle: inhibition of ovulation, alteration of the endometrium to impair implantation of the ovum, and alteration of the cervical mucus so that it becomes hostile to passage of spermatozoa (4).

First approved for use in the United States in 1960, oral contraceptives (OC) were used by an estimated 8.5 million American women and by 18.5 million women in the world in 1969 (5). That the number of OC users in both developed and developing portions of the world has substantially increased since 1969 is likely.

Within the last several years a number of undesirable side effects have been associated with OC use (4). Menstrual alterations reported include amenorrhea, delayed menses, pelvic cramps and spotting, together with a feeling of abdominal fullness, breast enlargement and/or tenderness, Physical changes including weight gain, weight loss, acne and chloasma have been cited. Complaints of gastric distress, nausea, vomiting, dizziness, headache, backache, leg cramps and unusual fatigue have been noted by women using OC. Psychological effects including anxiety, depression, nervousness and changes in libido have also been reported. Of considerable concern are the vascular effects of thromboembolism and hypertension. Alterations have also been reported in lipid, carbohydrate and protein metabolism as well as the metabolism of some vitamins and minerals. OC users have been reported to show elevated plasma vitamin A levels (6), reduced serum and whole blood folate levels (7), reduced plasma ascorbic acid (8), increased serum copper (9) and increased serum iron and serum total iron-binding capacity (10). Of all the vitamins investigated, vitamin B_6 has thus far been the most

intensely studied in women using OC.

Vitamin B_6 is a collective term for several forms of the vitamin: pyridoxal, pyridoxine, pyridoxamine and their 5-phosphate esters (11). Pyridoxal phosphate (PAL-P) is the active, coenzymatic form of vitamin B_6 and functions in numerous enzymatic reactions involving carbohydrate, lipid and protein metabolism. Some of these reactions have been used to indirectly judge vitamin B_6 nutritional status of individuals and to quantitatively assess their need for this vitamin. A frequently used method is the measurement of the urinary excretion of certain metabolites of the vitamin B_6 dependent tryptophan to nicotinic acid pathway after the consumption of a load dose of tryptophan. Vitamin B_6 deficient subjects have shown markedly elevated urinary excretion of xanthurenic acid (XA), kynurenine (KYN) and 3-hydroxykynurenine (3-OH-KYN) (12).

Evidence of altered tryptophan metabolism associated with OC use has been reported as early as 1966 by Rose (13) who found significantly elevated urinary excretion of XA after a load dose of tryptophan in women using the combined type of preparation. In a follow-up study Rose (14) detected increased excretion of 2 other tryptophan metabolites: 3-OH-KYN and 3-hydroxyanthranillic acid (3-OH-ANTR). Treatment with 20 to 40 mg of pyridoxine per day reduced the levels of these metabolites to those observed in control women. Similar observations have been made by others (15-20).

As elevated levels of tryptophan metabolites occur with the use of OC and also in vitamin B_6 deficiency, and because they can both be normalized by giving doses of pyridoxine hydrochloride (PIN-HCl), the conclusion has been drawn that a subclinical deficiency of vitamin B_6

exists in women using OC and could thus indicate an increased requirement for the vitamin by this group of women.

The studies of Rose et al. (18) and Brown et al. (19) indicated that the estrogenic component of the OC preparation was responsible for altering tryptophan metabolism. A more recent study by Rose et al. (21) suggests that although progestogenic steroids used alone have no effect, the nature of the progestogen used in the OC combination might modify the estrogen - induced abnormality in tryptophan metabolism. Rose and Adams (20) showed that the low dose (0.05 mg) estrogen containing OC combination, when used for 6 months or longer, resulted in increases in urinary excretion of tryptophan metabolites that were similar to the increases found in subjects using high dose (0.100 to 0.075 mg) estrogen containing OC. The authors concluded that estrogens, even at the lower dosage, used for prolonged periods of time precipitated abnormalities in tryptophan metabolism. These abnormalities were completely reversed by treatment with 20 mg of PIN-HCl daily for 1 month.

Vitamin B_6 concentrations in whole blood (22, 23), serum (24), plasma (25) and erythrocytes (26) have been utilized successfully as indices of vitamin B_6 nutriture. Vitamin B_6 in all blood fractions fell during vitamin B_6 depletion and rose upon supplementation of subjects with the vitamin. Only 3 studies investigating blood levels of vitamin B_6 in OC users appear in the literature. Brown et al. (27) examined 16 OC users and 9 nonusers fed a diet containing about 0.2 mg of vitamin B_6 for 1 menstrual cycle, then supplemented daily with 0.8, 2.0 or 20.0 mg of PIN-HCl for a second cycle. The OC users initially had slightly lower PAL levels, and after 1 week of depletion had significantly lower PAL levels than controls. Supplementation with 0.8 mg of PIN-HCl did

not restore PAL to starting values but 2.0 mg or 20 mg supplements elevated PAL levels above normal.

Bennink and Schreurs (28), however, found no alteration in blood vitamin B_6 due to OC usage for a minimum of 3 months in a group of 50 women. The mean blood vitamin B_6 level of this group was 17.6 ng/ml, with a range of 13.3 to 21.3 ng/ml. The accepted normal range for blood vitamin B_6 was 13.5 to 25 ng/ml, thus, the blood values for the OC users were considered normal.

A very recent study by Lumeng et al. (29) has investigated the effect of OC usage on plasma PAL-P levels with respect to age of the subject. The mean plasma PAL-P (7.8 ng/ml) for 55 OC users was significantly lower than the mean for 77 controls (9.4 ng/ml). When broken down into age groups, mean plasma PAL-P in the OC users of the 25 to 29 year and 30 to 34 year age groups was significantly lower than in age matched controls. However, no significant difference was detected between OC users and controls in the 20 to 24 year age group. Lumeng et al. (29) also demonstrated that an initial decrease in plasma PAL-P occured during the first 3 months of OC therapy in a group of 10 women, but that by 6 months only 3 had low levels. Thus, there is some indication that vitamin B_6 in the blood could be affected by age and duration of therapy.

Measurement of the activity of 2 vitamin B_6 dependent erythrocyte enzymes - alanine aminotransferase (E-Ala-AT) and aspartate aminotransferase (E-Asp-AT) in controlled vitamin B_6 depletion studies (22, 26, 30, 31) generally showed a decline in enzyme activity as depletion progressed and an increase upon vitamin B_6 supplementation. Baysal et al. (22) and Cinnamon and Beaton (31) found the E-Ala-AT system to be more sensitive to vitamin B_6 deficiency whereas Raica and Sauberlich (30)

and Donald et al. (26) were able to detect only small amounts of E-Ala-AT activity. Determination of percent in vitro PAL-P stimulation of aminotransferase activity provided a suitable indicator of vitamin B_6 status (30). The effect of OC usage on the activities of these two enzyme systems has been investigated since 1970. Doberenz et al. (32) examined E-Ala-AT activity and percent stimulation with in vitro PAL-P in 24 female subjects, 13 of whom were using OC of various types and for varying lengths of time. Mean basal activity of E-Ala-AT in OC users was 90.4 µg of pyruvate/ml of packed cells/hr whereas the nonusers showed a significantly higher ($P < 0.02$) mean value of 176.9 µg. Mean percent stimulation with PAL-P was significantly higher ($P < 0.001$) in the group of OC users (117.6%) when compared to the nonusers (22.4%). The authors concluded that their data supported the supposition of vitamin B_6 deficiency in women using OC.

Aly et al. (16) studied both E-Ala-AT and E-Asp-AT in a group of 5 women using OC and 5 women not using OC. Mean E-Ala-AT activity in this study was the same for both groups (6.2 µmoles NAD/ml of packed cells/hr). Percent stimulation with in vitro PAL-P was not determined. E-Asp-AT activity, however, was significantly elevated ($P < 0.01$) in the group of OC users (45.9 µmoles) when compared to the nonusers (34.3 µmoles). The percent stimulation was similar in both groups: 35.3% for the OC users and 32.6% for the nonusers.

Rose et al. (18) determined E-Ala-AT and E-Asp-AT activities in 16 women before they had used OC and after they had used them for 3 to 6 months. The enzyme activities were also determined in 34 women who had used OC for 6 to 36 months. No significant differences were observed in E-Ala-AT and percent stimulation between either group of OC users

and controls. Basal E-Asp-AT in the group using OC for more than 6 months (25.9 μg oxaloacetate/mg hemoglobin/hr) was significantly elevated ($P < 0.01$) when compared to E-Asp-AT in the OC users of 3 to 6 months (20.1 μg) or the controls (22.0 μg). Percent stimulation was not significantly different between groups.

In the following year Rose et al. (33) again studied E-Ala-AT and E-Asp-AT activity in 80 OC users, and in addition, examined the effect of PIN-HCl supplementation on aminotransferase activity. As was previously shown (16, 18) mean basal E-Ala-AT activity in the OC users was not significantly different from the mean activity in control women. The mean percent stimulation, however, was significantly elevated ($P < 0.025$) in the OC users when compared to the 50 controls, thus confirming the findings of Doberenz et al. (32). Basal E-Asp-AT activity was once again found to be significantly elevated ($P < 0.05$) in OC users compared to controls, no significant differences were observed in percent stimulation with PAL-P. A daily supplement of 40 mg of PIN-HCl was given to 20 OC users for 4 weeks and to 10 others for 8 weeks. No rationale for the dosage used by Rose et al. (33) was stated. The results of the effects of this supplementation on aminotransferase activity appears in Table 1. Supplementation for 4 or 8 weeks showed significant elevation in basal activity of both aminotransferases and corresponding reductions in percent stimulation by PAL-P.

A recent study by Salkeld et al. (34) examined the effect of OC usage on E-Asp-AT in relation to the duration of therapy and the estrogen content in a large sample of 233 women. The ratio of the increased activity (with PAL-P) to the original activity (without added PAL-P) gave an activation coefficient α E-Asp-AT which was used to assess

Table 1. Effect of pyridoxine administration[a] on erythrocyte aminotransferases. Rose et al. (33)

		Alanine Aminotransferase		Aspartate Aminotransferase	
		Activity[b]	Stimulation (%)	Activity[c]	Stimulation (%)
Group 1	A) before PIN-HCl	1.18±0.4	22±17	24.5±4.7	67±17
	B) after PIN treatment for 4 weeks	2.38±0.99[d]	8±5[d]	36.8±4.6[d]	22±5[d]
Group 2	A) before PIN-HCl	1.04±0.69	30±17	22.6±6.2	73±19
	B) after PIN treatment for 8 weeks	3.07±1.49[d]	8±5[d]	47.1±9.5[d]	18±7[d]

a Pyridoxine - hydrochloride (PIN-HCl), 40 mg daily.

b Expressed as mg sodium pyruvate/µg hemoglobin/hr.

c Expressed as µg oxaloacetate/mg hemoglobin/hr.

d Differs significantly from pretreatment value $P < 0.001$.

vitamin B_6 status. From 300 normal blood donors, an αE-Asp-AT of $\leq$1.64 was considered to be normal; 1.65 to 1.69 was considered marginal; and $\geq$1.70 was considered deficient. In the sample of OC users, mean αE-Asp-AT was 1.64, compared to a mean of 1.50 for the control group. No significant difference in αE-Asp-AT was evident between the users of low dose estrogen containing OC and the high dose estrogen containing OC (1.64 and 1.62, respectively). Duration of use had no significant influence. The authors also made the observation that an intake of 10 to 15 mg of PIN-HCl by a few women in the OC group was insufficient to normalize αE-Asp-AT. Because this was self-medication in the form of a multi-vitamin pill and not a supplement given under controlled conditions, the reliability of such an assumption is questioned.

Several parameters used to assess vitamin B_6 nutriture have indicated possible depletion of the vitamin in some women using OC, therefore attempts have been made to determine the degree of depletion and to establish a requirement which would meet this increased need for vitamin B_6. Luhby et al. (17) found that daily PIN-HCl supplements of 2 or 5 mg during a complete menstrual cycle were insufficient to normalize XA excretion after a 1 g oral load of tryptophan. Supplementation with the vitamin at the 10 mg level normalized XA excretion in 75% of the OC users, and at the 20 mg level, 14% still excreted elevated XA levels. Using regression analysis a daily PIN-HCl supplement of 25 mg was calculated as being necessary to normalize XA excretion in "all" subjects. With a safety factor of 5 mg added on to the minimum of 25 mg, the daily dose recommended by Luhby et al. (17) for all women using OC is 30 mg.

Price et al. (35) demonstrated that some women using estrogen containing OC spontaneously excreted elevated levels of 3-OH-ANTH,

which was reversed by daily administration of 20 mg of PIN-HCl. Rose et al. (18) supplemented 20 mg of PIN-HCl daily to 4 subjects using OC which resulted in a decrease in tryptophan metabolite excretion and a markedly increased basal activity of E-Asp-AT and E-Ala-AT with corresponding decreases in percent stimulation. Leklem et al. (36) investigated tryptophan metabolism in 16 OC users on a low vitamin B_6 diet (0.2 mg vitamin B_6/day) for 1 menstrual cycle and then repleted with either 0.8, 2.0 or 20.0 mg PIN-HCl daily for an additional cycle. Abnormalities occurring in excretion of tryptophan metabolites after a load dose were not corrected by either 0.8 or 2.0 mg of vitamin B_6 per day, whereas 20.0 mg per day reversed the abnormalities in 5 of 16 subjects within 1 week of repletion. The authors concluded that 0.8 or 2.0 mg of the vitamin was not enough to correct altered tryptophan metabolism in OC users. Brown et al. (27) examined plasma PAL levels in the same group of OC users and found that 2.0 mg of PIN-HCl daily elevated plasma PAL levels above normal. Adams et al. (37) suggested that 40 mg of vitamin B_6 was necessary to alleviate the psychological side effects experienced by some OC users.

Studies investigating OC - induced alterations in tryptophan metabolism indicate rather consistently that 20 to 30 mg of vitamin B_6 daily will correct these abnormalities. Salkeld et al. (34) pointed out, however, that whereas 80 to 100% of OC users are known to exhibit abnormal excretion of tryptophan metabolites, only 50% of his sample showed evidence of reduced E-Asp-AT activity. Lumeng et al. (29) found abnormally low plasma PAL in only 4 of 11 subjects who demonstrated abnormally high XA excretion.

Researchers who have used parameters other than those measuring levels of tryptophan metabolites in urine have found that much lower doses of vitamin B_6 (0.8 to 2.0mg) restored the reduced plasma PAL in OC users (27)

and that doses in the range required to correct abnormal tryptophan metabolism can cause significant and perhaps undesirable elevations in E-Ala-AT and E-Asp-AT activities (33). Estrogen administration alone is known to cause enzyme induction of alanine and tyrosine aminotransferase in rat liver (38), a phenomena which could account for the decline in plasma tyrosine levels noted in OC users (39). Total plasma amino acid levels are also known to be reduced in women using OC (40). Rose et al. (33) has expressed the concern that if cofactor induction of apoenzyme described by Greengard and Gordon (41) occurs and is superimposed on the estrogen-mediated increase of amino acid catabolizing enzymes, a further decrease in plasma amino acids might occur. Such an effect could enhance amino acid deficiencies in areas where protein malnutrition already exists, and where, ironically, the pill could be used to great advantage in population control.

Previous workers (17, 36) have reported that PIN-HCl supplements equivalent to the Recommended Dietary Allowance (RDA) (USA) (11) of 2.0 mg per day were insufficient to decrease the abnormally high XA excretion found in OC users, but a level of at least 20 mg of PIN-HCl was effective. The present study was designed to use a multiparameter approach; incorporating tryptophan metabolites, blood and urine levels of vitamin B_6, urinary excretion of 4-pyridoxic acid and erythrocyte aminotransferase activity to:

1) assess the vitamin B_6 nutritional status of young women using OC,
2) to determine whether young women using OC required more vitamin B_6 than nonusers, and
3) if the requirement was greater, to establish what this requirement

might be.

Results obtained from three parameters to assess vitamin B_6 requirement; E-PAL, E-Ala-AT and E-Asp-AT are reported here. Results from the other parameters, including urinary excretion of vitamin B_6 and 4-pyridoxic acid and urinary excretion of the tryptophan metabolites - XA, KYN, 3-OH-KYN, and kynurenic acid are not included in this thesis.

EXPERIMENTAL PROCEDURE

Subjects

Sixteen female university students participated in the study. Eight of the young women using OC for the purpose of birth control served as the experimental group. The remaining 8 were not using OC and thus served as the control group. A preliminary questionnaire facilitated the selection of suitable candidates for the study and a complete medical examination by a university physician ensured that all subjects were in good physical health and had normal liver function.

Some of the general characteristics of the subjects are shown in Table 2. They ranged in age from 18 to 23 years. The mean age for the experimental group was 20 years with a mean age of 21 years for the control group. Initial weights for the experimental group varied from 120 to 153 pounds with a mean of 139 pounds. The control group had a similar range from 125 to 150 pounds, the mean being 135 pounds. Both groups had normal menstrual histories.

The name, dosage, and relative potency of the OC used, as well as the duration of use are shown in Table 3. Seven of the eight subjects used the combined type of contraceptive pill; whereas one of the group used the sequential type. Seven of the eight subjects had been under hormonal therapy for at least 3 months, while one subject, F, had begun hormonal therapy just prior to the study.

Diet

A diet of natural foods, adequate in calories and protein and low in vitamin B_6 was used throughout all phases of the study. The basal diet (see Appendix I) provided 1650 kcal, 65 gm protein, 670 mg calcium, 7.9 mg iron, 0.42 mg thiamine, 0.82 mg riboflavin, 7.02 mg niacin,

Table 2. General characteristics of the subjects participating in the study.

Subject	Age[a]	Height[b]	Weight[c]	Menstrual History		
				Age of onset[d]	Length of cycle[e]	Length of flow[e]
Experimental Group						
A	19	5-2	120	13	25	5
B	21	5-4	124	13	28	6
C	18	5-5	135	14	28	4
D	20	5-4	139	13	28	5
E	21	5-7	145	13	28	5
F	20	5-4	145	12	29	5
G	22	5-5	150	15	28	5
H	22	5-8	153	14	34	5
Mean values:	20	5-4	139	13	28	5
Control Group						
I	20	5-5	125	13	30	4
J	21	5-1	128	13	26	4
K	22	5-6	129	14	32	5
L	23	5-6	130	13	28	6
M	19	5-5	134	12	27	5
N	19	5-1	135	13	28	6
O	22	5-8	147	12	29	6
P	20	5-8	150	14	29	6
Mean values:	21	5-3	135	13	29	5

a Values expressed in years to the nearest six months.

b Values expressed in feet plus inches without shoes.

c Values expressed to the nearest pound at the start of the study.

d Values expressed in years.

e Refers to the ovulatory cycle. Values expressed in days.

Table 3. Names and dosages of the oral contraceptive preparations used by the subjects.

	ESTROGEN				PROGESTOGEN			
Subject	Commercial Name	Name	Dosage[a]	Potency[b]	Name	Dosage[a]	Potency[c]	Duration of Therapy[d]
A	Orthonovum 1/2	Mestranol	0.10	2.0	Norethindrone	0.50	0.5	12
B	Ovral	Ethinyl Estradiol	0.05	1.8	D-norgestrel	0.25	15.0	13
C	Orthonovum 1/50	Mestranol	0.05	1.0	Norethindrone	1.00	1.0	25
D	Ovral	Ethinyl Estradiol	0.05	1.8	D-norgestrel	0.25	15.0	6
E	Ovral	Ethinyl Estradiol	0.05	1.8	D-norgestrel	0.25	15.0	7
F	Ovral	Ethinyl Estradiol	0.05	1.8	D-norgestrel	0.25	15.0	1
G	Norinyl-1	Mestranol	0.05	1.0	Norethindrone	1.00	1.0	3
H	Norquen (Sequential)	14 days - mestranol	0.08	1.6	----------	---	-----	--
		7 days - mestranol	0.08	1.6	Norethindrone	2.0	2.0	14

a Expressed in mg.

b Relative to 0.05 mg mestranol as a standard unit of 1.0.

c Relative to 1.0 mg norethindrone as a standard unit of 1.0.

d Expressed in months.

4,290 IU vitamin A, and 73 mg ascorbic acid per day. Nutrient estimations were based on USDA Handbook No. 8 (42). Calcium, iron, thiamine and riboflavin were supplemented in capsule form so that the total intake of these nutrients reached the RDA (USA) (11) suggested for this age group (see Table 4).

The basal diet was similar in composition to one used in a previous metabolic study which was analysed to contain 0.34 mg of vitamin B_6 per day (43). The basal diet used in this study was modified by the addition of 2 extra slices of bread per day, containing 0.01 mg of vitamin B_6 per slice and 20 g extra apple jelly. The resulting total daily intake of the vitamin from the basal diet would be approximately 0.36 mg.

Some ad libitum foods, virtually devoid in vitamin B_6, were included to provide additional calories for maintenance of initial body weight (see Appendix I). These foods included butter, sugar, shortbread cookies, hard candy, mints, butter-crunch and gingerale. A daily record was kept of the amounts eaten so that total caloric intake could be calculated. Towards the end of the study, dried herbs and spices were added to some of the foods, and the combination of the ingredients was varied to avoid monotony. These changes were made without significant alteration of the daily basal intake.

Experimental Outline

All meals were prepared in the metabolic unit, and were consumed there. Occasionally, when necessary, a meal was packaged and consumed at home. A daily record was kept by each subject, whereby unusual activity, hours of sleep, ingestion of aspirin or other medications, or any other factor which might influence biochemical results was noted.

Table 4. Daily nutrient intake from the basal diet plus vitamin and mineral supplements per subject.

Nutrient		Basal Diet	Supplement	Total Intake	RDA (USA) (11)
Kcalories		1650	-	1650	2000
Protein	g	65	-	65	55
Carbohydrate	g	188	-	188	
Fat	g	73	-	73	
Calcium	mg	670	210	880	880
Phosphorous	mg	830	-	830	800
Iron	mg	7.9	10.6	18.5	18
Thiamine	mg	0.42	0.66	1.08	1.0
Riboflavin	mg	0.82	0.80	1.62	1.5
Niacin	mg	7.02	-	7.02	13
Ascorbic Acid	mg	73	-	73	55
Vitamin A	IU	4290	-	4290	5000
Vitamin B_6	mg	0.36	-	0.36	2.0

The study consisted of three phases. The first 10 days constituted the pre-depletion phase, when both experimental and control subjects consumed the weighed basal diet plus a vitamin B_6 supplement of 1.70 mg/day. This brought the total daily intake of the vitamin to 2.06 mg, thus meeting the RDA (USA) for vitamin B_6 (11). The pre-depletion phase permitted the subjects to become familiar with the diet and the metabolic routine. In addition, normal levels of the biochemical parameters to be studied would be established in both control and undepleted experimental subjects.

Only the experimental subjects underwent the depletion and repletion phases. The depletion phase began on day 11 when the vitamin B_6 supplement of 1.7 mg/day was withdrawn, thus resulting in a net daily intake of the vitamin of 0.36 mg. Depletion continued for 32 days.

Repletion of the subjects was executed in 3 steps. On day 43 a PIN-HCl supplement of 0.6 mg/day was given, bringing the total daily intake to 0.96 mg. This level of intake was continued for 8 days. On day 51 the diet was supplemented with 1.2 mg of the vitamin, now bringing the total daily intake to 1.56 mg. This was continued for 9 days until the final level of repletion was instituted. On day 60, 4.7 mg of PIN-HCl was administered daily and continued for 7 days, bringing the total daily intake of vitamin B_6 to 5.06 mg.

Collection and Storage of Blood Samples

Fasting blood samples of approximately 14 ml each were withdrawn by venipuncture after 1, 10, 17, 24, 31, 38, 42, 49, 58 and 65 days on the study. Vacutainer tubes containing 0.07 ml of 15% EDTA were used. Duplicate analysis for microhematocrit and hemoglobin were done on fresh

whole blood from all subjects each time blood was taken (see Appendix II).

Plasma and erythrocytes were separated by centrifugation. The erythrocytes were prepared for analysis of E-PAL, E-Ala-AT and E-Asp-AT (see Appendix III). All samples were tightly stoppered, sealed with tape and stored at -20° C. Those samples to be used for analysis of vitamin B_6 were protected from light.

Methodology

A. Erythrocyte vitamin B_6 level

The determination of vitamin B_6 in the erythrocyte involved extraction of the vitamin from the tissue, chromatographic separation of B_6 vitamers, and finally, microbiological assay of the vitamin.

An acid hydrolysis of the erythrocyte hemolysate was required to liberate vitamin B_6 from phosphate complexes and protein binding (44). Extraction was achieved by a combination of acid hydrolysis with 10% TCA followed by autoclaving at 21 lb. pressure (45) (see Appendix IV. A. 9). The resulting hydrolysate was then applied to a column containing 6 mls of activated Dowex (50 AG WX8) ion exchange resin according to the method of Toepfer and Lehmann (46) (see Appendix IV. A. 11), and the pyridoxal fraction eluted (see Appendix IV. B). This method facilitates the chromatographic separation of the three B_6 vitamers pyridoxal, pyridoxine, and pyridoxamine. Kelsay et al. (23) have established that the major constituent of total erythrocyte vitamin B_6 is pyridoxal, thus, pyridoxal was the only fraction eluted.

The microbiological procedure of Atkin et al. (47) as modified by Storvick et al. (44) was used to determine the pyridoxal content of the column eluates (see Appendix IV. C). Saccharomyces carlsbergensis was

the assay organism. A standard solution of pyridoxal was chromatographed simultaneously with the erythrocytes (see Appendix IV. B). Hydrolysing the standard had no beneficial effect on growth of the organism, thus this step was omitted. Vitamin B_6 in the samples was interpolated from standard curves obtained from assaying the chromatographed standard.

All procedures were carried out in semi-darkness, using a red light for necessary illumination, as vitamin B_6 is subject to destruction when exposed to light (46).

B. Erythrocyte Alanine Aminotransferase

The determination of E-Ala-AT activity was based on the colorimetric procedure of Heddle et al. (48) for whole blood and modified for erythrocytes by Woodring and Storvick (49). In vitro percent stimulation by pyridoxal phosphate was determined according to Woodring and Storvick (49). One additional change was made in the present study: 2 ml of 2.5% alcoholic potassium hydroxide was used rather than the 1 ml used by Woodring and Storvick (49).

The procedure involves the formation of pyruvate as the end product of the transamination reaction between α-ketoglutarate and alanine. Pyruvate reacts with 2, 4-dinitrophenylhydrazine to form the hydrazone which is quantitatively extracted with toluene, and color developed in alcoholic potassium hydroxide. Optical density of this colored complex was measured in a Beckman DU-2 spectrophotometer at 490 mμ. The optical density of the colored complex is proportional to the amount of pyruvate formed, thus indicating the activity of E-Ala-AT. A standard curve prepared from solutions providing 10 to 80 μg of sodium pyruvate per tube (see Appendix V) was used to determine the amount of pyruvate formed by the erythrocyte samples.

C. Erythrocyte Aspartate Aminotransferase

E-Asp-AT activity was determined spectrophotometrically using a premeasured kit[a]. The procedure followed was similar to that described by Fu (43) with the exception that 0.5 ml of the original erythrocyte hemolysate (see Appendix III) was diluted with 4.5 ml of distilled water, resulting in a 50X dilution of the original packed cell volume, rather than the 25X dilution used by Fu (43).

The determination is based on the following reactions as described by Karmen (50).

1) α-Ketoglutarate + L-aspartate $\overset{\text{Asp-AT}}{\rightleftharpoons}$ glutamate + oxaloacetate

2) oxaloacetate + NADH + H^+ $\overset{\text{MDH}^b}{\rightleftharpoons}$ malate + NAD^+

The aminotransferase reaction 1) is coupled to the reduction of oxaloacetate by NADH + H^+ 2) in the presence of MDH. The rate of NADH + H^+ oxidation is a function of the rate of transamination and thus, the rate was monitored by the measurement of the rate of disappearance of NADH, or, the rate of decrease in light absorption at 340 mμ, where the reduced pyridine nucleotides have maximum absorption (50).

The pyridoxal phosphate stimulated activity of E-Asp-AT was determined according to the method of Cheney et al. (51).

D. Analysis of data

Individual values, mean, and standard error of the mean are presented for each parameter measured. Doubtful observations were rejected according to the Q-test described by Harris and Kratochvil (52).

A paired t-test was used to determine the levels of significance of

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[a] Boehringer Mannheim Company.

[b] Malic dehydrogenase.

the differences in the values of E-PAL and transaminase activities after depletion and at the various stages of repletion in the experimental subjects. An unpaired t-test was used to test the significance of differences between the values of the experimental group and those of the control group.

RESULTS AND DISCUSSION

Pre-study clinical analyses

The pre-study clinical examination showed all subjects to be in good health. Results of the analyses conducted are shown in Table 5.

Hemoglobin levels were determined by a cyanmethemoglobin method. Both control and experimental groups in this study had hemoglobin levels within the normal range of healthy adult women (53). Although the experimental group had a slightly lower level than did the control group, no significant difference was evident between the two groups. Fisch (54), in an examination of red blood cell indices in 3736 women found a slight but statistically significant decrease in hemoglobin and hematocrit levels and a decrease in red blood cell count in women using OC.

Liver function was assessed by the determination of serum aspartate aminotransferase (S-Asp-AT) activity. An elevated level of S-Asp-AT is indicative of liver cell damage as a result of the release of this enzyme from liver cells when injured (55). The S-Asp-AT levels of all subjects in this study were considered to be normal by the examining medical personnel. Several investigators have observed elevated levels of serum aminotransferases in women taking OC (56, 57). Other workers have found that these slight increases in serum aminotransferase levels usually occurred and disappeared within 1 to 3 months of continued therapy (58, 59), thus suggesting that impairment of hepatic function is clinically insignificant and transient. Swaab (60) and Haehn et al. (61) in separate studies found no significant elevations in S-Asp-AT activity in women taking OC, regardless of duration of therapy.

The presence of urinary glucose was investigated because of reports that OC precipitate impaired glucose tolerance in some women using these

Table 5. Results of the pre-study clinical analyses.

Subject	Hemoglobin Level[a]	S-Asp-AT[b,c]	Urinary Glucose[d]
Experimental group			
A	12.5	N	-
B	13.2	N	-
C	15.2	N	-
D	12.8	N	-
E	13.2	N	-
F	12.4	N	-
G	12.8	N	-
H	13.1	N	-
Mean value	13.2		
SEM[e]	±0.3		
Control group			
I	13.4	N	-
J	14.2	N	-
K	13.5	N	-
L	12.7	N	-
M	15.0	N	-
N	14.8	N	-
O	14.5	N	-
P	15.6	N	-
Mean value	14.2		
SEM[e]	±0.3		

a Expressed as gm/100 ml blood.

b Serum aspartate aminotransferase.

c N indicates normal.

d - indicates negative.

e Standard error of the mean.

preparations (62-64). All participants in the study showed no evidence of excess urinary glucose.

Study Analyses

A. Hemoglobin and Hematocrit

Hemoglobin and hematocrit levels in the experimental group were determined after 1, 10, 17, 24, 31, 38, 42, 49, 58 and 65 days on the study; and after 1 and 9 days on the study in the control group. The results of these analyses are presented in Tables 6, 7 and 8.

There was relatively little fluctuation in either parameter during the three phases of the study, when varying levels of vitamin B_6 were consumed. Both hemoglobin and hematocrit values obtained in the study compared favorably with those obtained in a similar study conducted with adult women who were not using OC (43).

There is evidence that a low vitamin B_6 diet may result in anemia (65). Horrigan and Harris (66) have described iron resistant, microcytic, hypochromic anemias that respond only to large doses of pyridoxine. The subjects showed no sign of anemia throughout the study, thus it appears that consumption of 0.36 mg vitamin B_6 per day for 32 days did not produce a severe enough deficiency to precipitate changes in hemoglobin and hematocrit levels.

The control group had slightly higher levels of both hemoglobin and hematocrit than the experimental group on day 2 of the study. This difference however, was not significant as judged by the unpaired t-test. After 9 days on an identical diet containing 2.06 mg of vitamin B_6 per day, both groups were found to have very similar levels of hemoglobin and hematocrit.

Table 6. Effect of vitamin B_6 intake on hemoglobin levels[a] of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	13.0	13.6	14.7	13.9	14.2	13.4	15.6	13.4	14.0±0.3
10	2.06	13.0	15.1	16.1	14.2	14.8	13.8	15.8	13.0	14.4±0.4
17	0.36	13.6	14.6	15.0	15.2	13.8	13.0	15.0	13.6	14.2±0.2
24	0.36	13.6	15.0	16.0	15.1	14.6	14.0	14.1	13.2	14.4±0.3
31	0.36	14.0	14.4	15.2	13.9	14.6	14.0	14.6	-	14.4±0.2
38	0.36	12.7	14.8	14.7	13.0	14.1	13.6	14.5	13.5	13.9±0.3
42	0.36	13.3	14.4	16.2	14.7	14.8	12.8	15.4	14.0	14.4±0.4
49	0.96[d]	14.2	13.9	15.2	13.9	14.2	14.6	13.7	13.7	14.2±0.2
58	1.56[e]	14.2	15.2	15.7	14.8	14.6	13.9	15.3	13.8	14.7±0.3
65	5.06[f]	12.6	13.2	15.4	13.0	14.0	13.6	13.6	11.9	13.4±0.4

a Expressed as g/100 ml of blood.

b Expressed as mg/day.

c Standard error of the mean.

d 0.6 mg PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg PIN-HCl was supplemented daily between day 60 and 66.

Table 7. Effect of vitamin B_6 intake on hematocrit levels[a] of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	38.0	42.0	41.0	41.0	42.0	39.5	44.0	38.5	40.8±0.7
10	2.06	37.0	41.0	44.0	40.0	42.0	38.0	44.0	36.0	40.2±1.8
17	0.36	39.0	40.0	42.0	43.0	40.0	39.0	41.5	39.0	40.4±0.5
24	0.36	38.5	41.5	45.0	42.0	41.0	40.5	40.5	38.0	40.9±0.8
31	0.36	40.0	41.0	42.5	41.0	42.5	41.0	42.0	-	41.4±0.9
38	0.36	36.0	40.0	40.5	37.5	39.5	39.5	41.0	38.0	39.0±0.6
42	0.36	37.0	40.0	43.0	40.5	41.0	38.0	42.0	39.0	40.1±0.7
49	0.96[d]	40.0	39.5	44.0	40.0	42.0	41.5	39.5	38.0	40.6±0.6
58	1.56[e]	39.5	42.5	43.0	40.0	42.5	40.0	43.5	37.0	41.0±0.8
65	5.06[f]	39.0	41.0	44.5	39.0	41.0	39.0	40.0	36.0	39.9±0.8

a Expressed as % packed RBC in volume of blood.

b Expressed as mg/day.

c Standard error of the mean.

d 0.6 mg PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg PIN-HCl was supplemented daily between day 60 and 66.

Table 8. Effect of a constant vitamin B_6 intake[a] on hemoglobin[b] and hematocrit[c] levels in 8 adult women not using oral contraceptives.

Days on Diet	Vitamin B_6 Intake	SUBJECTS								Mean ±SEM[d]
		I	J	K	L	M	N	Q	P	
Hemoglobin[b]										
1	2.06	13.4	15.0	15.6	13.4	17.4	14.8	16.2	15.6	15.2±0.4
9	2.06	11.0	-	14.1	14.1	16.4	14.2	15.4	14.8	14.3±0.6
Hematocrit[c]										
1	2.06	37.0	41.5	42.5	38.0	46.0	43.0	46.0	42.0	42.0±1.2
9	2.06	33.5	-	42.0	41.0	46.5	42.0	44.5	42.0	41.6±1.5

a 2.06 mg of vitamin B_6 was consumed daily for 10 days.

b Values expressed as g/100 ml of blood.

c Values expressed as % packed RBC in volume of blood.

d Standard error of the mean.

B. Erythrocyte Pyridoxal Concentration

The effect of vitamin B_6 intake on the E-PAL concentration of 8 adult women using OC is presented in Table 9 and Figure 1. The E-PAL values obtained for this group of OC users during all phases of depletion and repletion ranged from 10 to 55 ng/ml of packed cells. The E-PAL concentration of the nonusers of OC (see Table 12), while consuming 2.06 mg of vitamin B_6, ranged from 20 to 41 ng/ml of packed cells. Although slightly higher, the E-PAL range for the nonusers of OC is in agreement with the E-PAL range of 13 to 31 ng/ml of packed cells reported by Baker et al. (67) for normal subjects. The method used by Baker was a microbiological method with Tetrahymena pyriformis as the assay organism. Preliminary analysis on whole blood from the nonusers of OC in the present study produced a range of 13 to 29 ng of PAL/ml of whole blood. Storvick et al. (68), Baysal et al. (22) and Kelsay et al. (23), all using the microbiological method with Saccharomyces carlsbergensis as the assay organism, reported PAL ranges in whole blood from normal subjects of 3.4 to 23.2 ng/ml, 5.4 to 15.1 ng/ml and 1.4 to 7.2 ng/ml, respectively. Thus, the values for pyridoxal concentration in erythrocytes and whole blood obtained for the nonusers of OC of the present study are comparable to others appearing in the literature.

Adjustment Period: Fasting blood samples were taken from each experimental subject after 1 day on the study to establish a basal erythrocyte level of PAL on an ad libitum intake. The mean E-PAL at this time was 34.6 ng/ml of packed cells. After 10 days of adjustment on a basal diet containing 2.06 mg of vitamin B_6/day, E-PAL dropped slightly to 28.7 ng/ml of packed cells. There was no significant difference between these two values.

The level of 28.7 ng/ml was chosen as the pre-depletion value because

Table 9. Effect of vitamin B_6 intake on the mean concentration of erythrocyte pyridoxal[a] in 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	27.9	28.1	38.9	40.1	47.4	-	37.4	22.2	34.6±3.3
10	2.06	27.3	25.4	29.3	22.5	27.0	37.6	28.9	31.3	28.7±1.6
17	0.36	54.8	22.9	19.2	25.7	55.8	39.1	49.9	36.1	37.9±5.1
24	0.36	37.8	34.2	41.6	24.7	31.3	36.8	37.1	37.9	35.2±1.8
31	0.36	21.1	28.2	35.8	29.4	28.3	16.3	30.8	-	27.1±2.4
38	0.36	14.9	12.8	18.9	28.0	21.6	17.4	16.8	13.5	18.0±1.8
42	0.36	11.7	14.4	15.8	18.3	13.2	11.0	17.4	10.1	14.0±1.1
49	0.96[d]	16.2	20.8	22.0	21.7	23.1	20.9	26.2	18.4	21.2±1.1
58	1.56[e]	32.3	28.7	26.4	28.8	32.0	29.0	35.0	26.5	29.9±1.1
65	5.06[f]	32.6	40.2	42.8	53.4	30.6	42.6	35.8	35.2	39.2±2.6

a Expressed as ng/ml packed RBC.

b Expressed as mg/day.

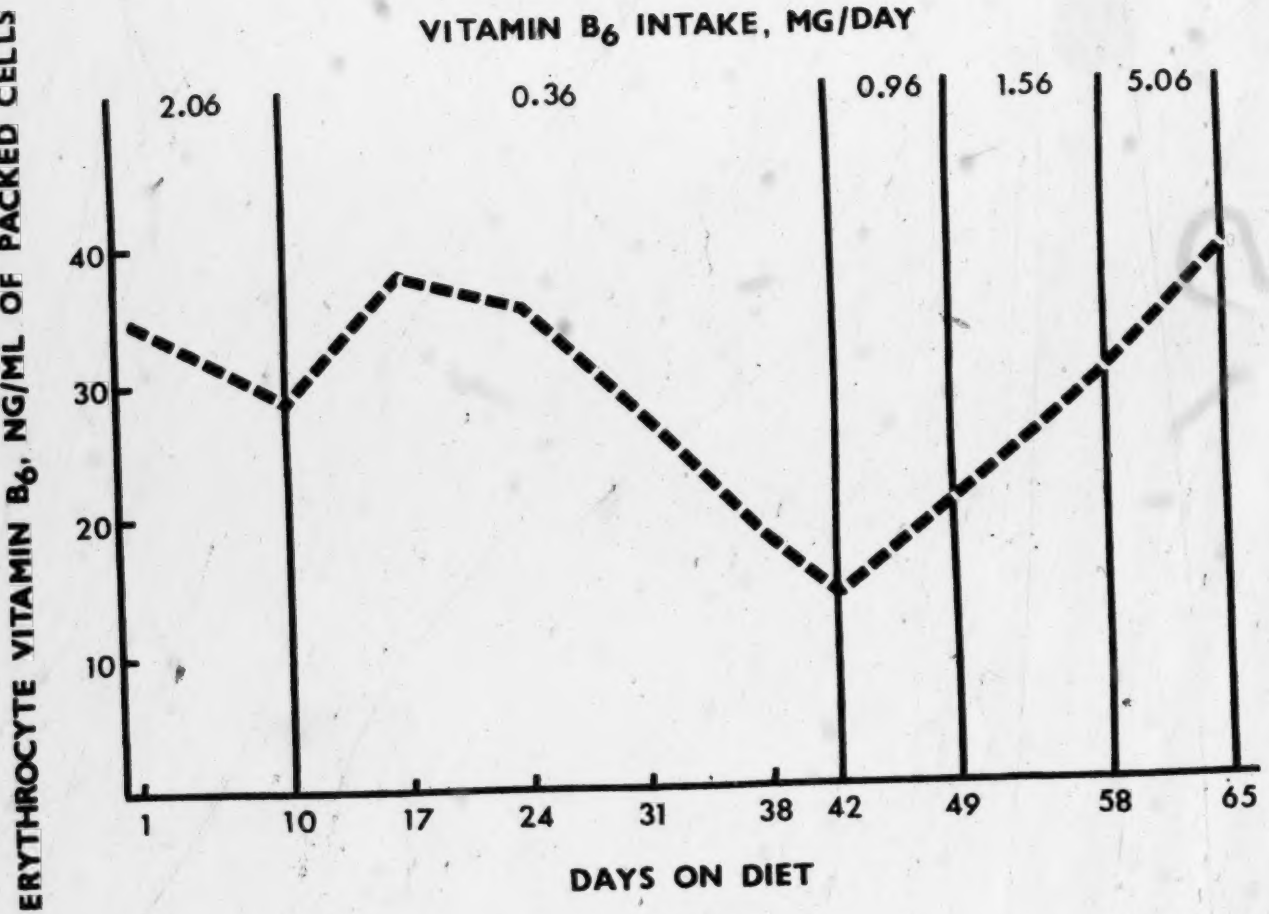
c Standard error of the mean.

d 0.6 mg PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg PIN-HCl was supplemented daily between day 60 and 66.

Figure 1. Effect of vitamin B_6 intake on mean concentration of erythrocyte pyridoxal in 8 adult women using oral contraceptives.


VITAMIN B6 INTAKE, MG/DAY
2.06
0.36
0.96
1.56
5.06
ERYTHROCYTE VITAMIN B6, NG/ML OF PACKED CELLS
40
30
20
10
1
10
17
24
31
38
42
49
58
65
DAYS ON DIET

presumably it was not influenced by previous ad libitum dietary intake, nor by unfamiliarity of the subjects with the study.

Depletion Period: After 7 days on the low vitamin B_6 diet (0.36 mg/day), the mean E-PAL concentration increased from 28.7 to 37.9 ng/ml packed cells, representing a 32% increase. This increase was attributable principally to 3 subjects (A, E and G) and was not statistically significant. Fu (43) observed a similar phenomena in a study (Appendix VII) in which young adult women consuming a low vitamin B_6 diet (0.34 mg/day) for 8 days had an increase of 18% in erythrocyte vitamin B_6 over the initial, pre-depletion value. This effect could be a mobilization of stores of vitamin B_6 from other body tissues to the erythrocyte in response to the stress of a diet low in the vitamin.

Following 2 weeks of consuming the low vitamin B_6 diet, the mean E-PAL concentration of the subjects dropped slightly to 35.2 ng/ml of packed cells, but still remained higher than the pre-depletion value of 28.7 ng/ml. Following 3 weeks of depletion, mean E-PAL dropped to 18 ng/ml of packed cells. This value was 63% of the mean value obtained on day 10; a decrease which was significantly different ($P < 0.01$). After 32 days of consuming the low vitamin B_6 diet, mean E-PAL dropped further to 14.0 ng/ml of packed cells, and therefore when the depletion phase was terminated, only 49% of the original E-PAL level was retained. The difference between the undepleted level and the most severely depleted level was significantly different ($P < 0.001$).

Fu (43) studied the effect of vitamin B_6 depletion and repletion on erythrocyte concentration of this vitamin in normal adult women of similar age as the subjects in the present study, but not using OC. The low vitamin B_6 diet used by Fu contained 0.34 mg of the vitamin per day

and was similar in composition to the diet used in the present study. A comparison of the changes in E-PAL that occured during depletion and repletion in the study by Fu and in the present study appears in Table 10 and Figure 2. A slight increase in E-PAL concentration occured after the first week of depletion in both studies. After 29 days on the deficient diet the nonusers of OC showed a decrease in mean erythrocyte vitamin B_6 levels to 47% of the original level. The OC users, after a similar period of depletion (28 days) also showed a decrease in E-PAL concentration, but only to 63% of the initial level. Fu's study continued for a further 14 days of depletion so that after 36 days on the low vitamin B_6 diet, mean erythrocyte levels dropped to 17% of the pre-depletion value, and after 43 days to 14% of the depletion value. Depletion in the present study continued for 32 days, at which time erythrocyte levels had decreased to 49% of the pre-depletion values.

It was originally intended to carry the depletion phase through at least 5 full weeks, based on the observation by Fu (43) that the erythrocyte levels of vitamin B_6 tended to level off after 5 weeks of depletion (see Figure 2). An additional week of depletion produced a further decrease of only 3% in mean erythrocyte vitamin B_6 concentration, indicating that maximum depletion had probably occurred at approximately 5 weeks. Furthermore, if the speculation that women using anovulatory steroids already had lower levels of erythrocyte vitamin B_6 proved to be correct, they could conceivably reach maximum depletion more rapidly than their non-treated counterparts. The depletion phase of the present study was terminated slightly earlier than planned, after kitchen personnel observed that several subjects had complained of feeling depressed and fatigued. Supplementation of the low vitamin B_6 diet with 0.6 mg/day

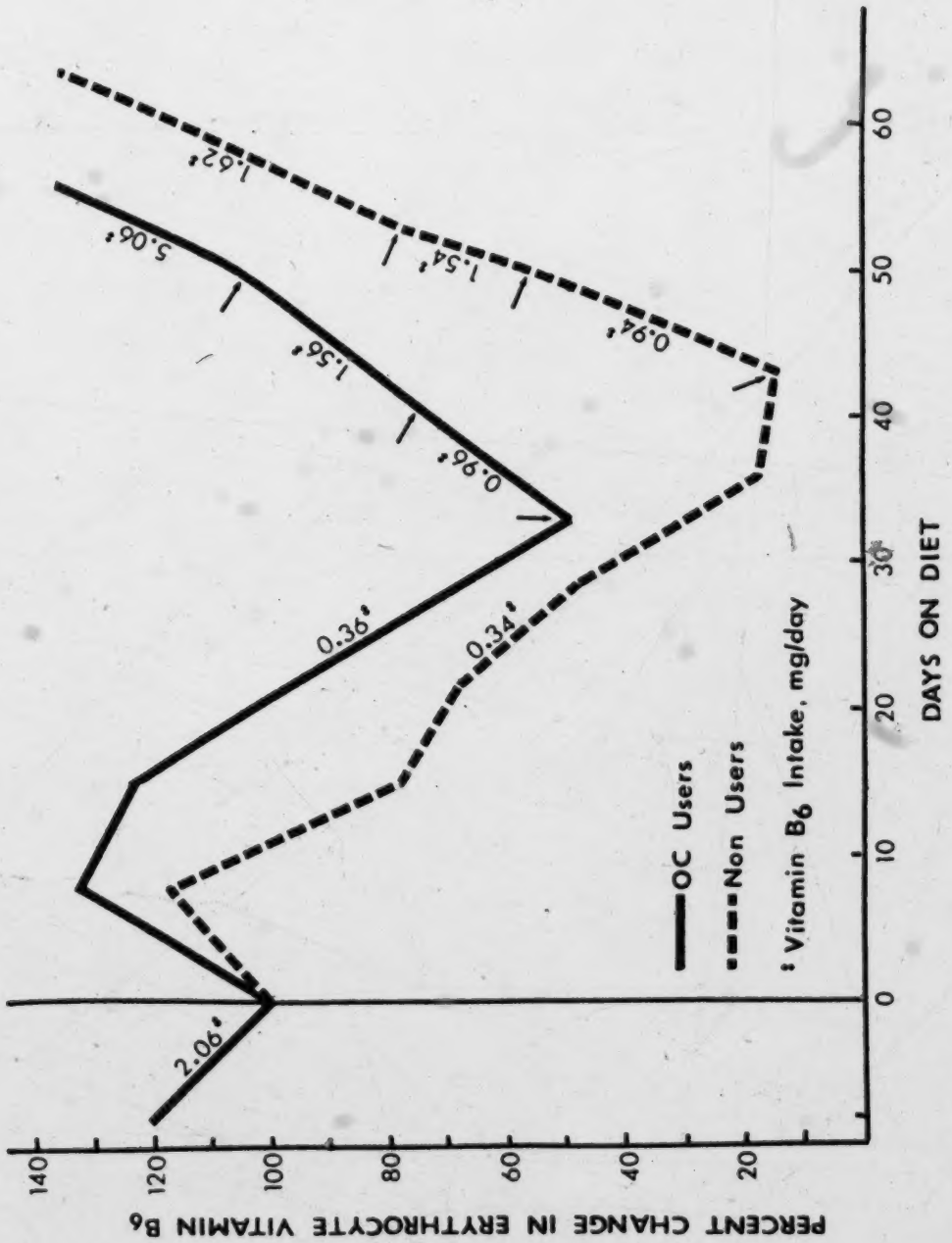
Table 10. Effect of vitamin B_6 intake on percent change in erythrocyte pyridoxal concentration in users and nonusers of oral contraceptives.

	Days on Diet	Vitamin B_6 Intake[a]	E-PAL[b]	% of Initial Level
	Adjustment 10 days	2.06	28.7	100
OC Users	7	0.36	37.9	132
	14	0.36	35.2	123
	21	0.36	27.1	94
	28	0.36	18.0	63
	32	0.36	14.0	49
	40	0.96	21.2	74
	49	1.56	29.9	104
	55	5.06	39.2	136
Non-Users of OC (43)	1	0.34	336	100
	8	0.34	397	118
	15	0.34	263	78
	22	0.34	230	68
	29	0.34	158	47
	36	0.34	58	17
	43	0.34	46	14
	50	0.94	188	56
	53	1.54	263	78
	64	1.62	454	135

a Expressed as mg/day.

b Expressed as ng/ml of packed cells.

Figure 2. Effect of vitamin B_6 intake on percent change in erythrocyte pyridoxal concentration in users and nonusers (43) of oral contraceptives.


PERCENT CHANGE IN ERYTHROCYTE VITAMIN B6
140
120
100
80
60
40
20
0
10
20
30
40
50
60
DAYS ON DIET
OC Users
Non Users
Vitamin B6 Intake, mg/day
2.06
0.36
0.96
1.56
5.06
0.34
0.94
1.54
1.62

of PIN-HCl was promptly started.

Although the degree of depletion achieved in this study was not as great as that achieved by Fu, the rates of change, as reflected by the slopes of the curves illustrated in Figure 2 are similar in both studies. Had the period of depletion been extended, a similar degree of depletion might have been obtained.

The effect of vitamin B_6 depletion on vitamin B_6 content of blood in adult men has been reported by Baysal et al. (22). They agree that plasma and whole blood vitamin B_6 levels also fall rapidly during vitamin B_6 depletion and rise following supplementation thus reflecting the level of intake. In the study by Baysal et al. (22) and Kelsay et al. (23), after 40 and 39 days respectively on an intake of 0.16 mg vitamin B_6 per day, E-PAL in male subjects decreased to 14% and 19% of the initial E-PAL, respectively. These levels of depletion agree with Fu's observations (43) but are somewhat lower than those obtained in the present study.

Repletion Period: After consuming the low vitamin B_6 diet for 32 days, the subjects were supplemented with 0.6 mg of PIN-HCl per day, bringing the total daily intake of the vitamin to 0.96 mg. This phase of repletion continued for days, and resulted in an increase of E-PAL in all subjects. The mean E-PAL concentration rose from 14.0 to 21.2 ng/ml of packed cells; the increase being statistically significant at a $P < 0.01$. E-PAL concentration at this level of supplementation reached 74% of the initial value. The difference between the undepleted erythrocyte concentration and the partially repleted erythrocyte concentration was significantly different ($P < 0.01$).

In the work of Fu (43), following 43 days on a diet containing 0.34 mg vitamin B_6 per day, supplementation of subjects with 0.6 mg PIN-HCl per day for 7 days increased E-PAL from 14% to 56% of the initial level. In the studies by Baysal et al. (22) and Kelsay et al. (23), following 40 and 39 days of depletion at a vitamin B_6 intake of 0.16 mg/day, supplementation with 0.5 and 0.6 mg of PIN-HCl increased blood concentrations of vitamin B_6 in male subjects from 14 to 39% and 19 to 47% of initial levels, respectively.

In the second repletion period, supplementation with 1.2 mg of PIN-HCl/day for 8 days significantly increased the mean E-PAL concentration from 21.2 ng/ml to 29.9 ng/ml of packed cells ($P < 0.001$). This supplement of 1.2 mg of PIN-HCl, resulting in a daily intake of 1.56 mg of vitamin B_6, restored levels of E-PAL to pre-depletion levels in 5 of the 8 subjects. Mean E-PAL concentration after this second phase of repletion increased to 104% of the pre-depletion value, with no significant difference between mean E-PAL concentrations after 10 and 58 days on the study. Thus, a daily intake of 1.56 mg of vitamin B_6 per day was sufficient to restore the mean E-PAL concentration to that obtained before depletion. However, this intake was inadequate to restore E-PAL levels to pre-depletion levels for 3 of the 8 subjects (C, F, H), who initially had the 3 highest E-PAL concentrations. This effect could not be attributed to the type of OC preparation used or the duration of use.

In the study conducted by Fu (43) a supplement of 1.2 mg/day for 3 days restored E-PAL levels to pre-depletion levels in 4 of the 8 subjects. Mean erythrocyte concentration rose to 78% of the initial value. The discrepancy in the rates of repletion between the present study and the study by Fu could be due to the fact that the second phase of repletion

in the present study was 8 days as opposed to 3 days in the study by Fu.

Subjects in the final repletion period received 4.7 mg of PIN-HCl/ day, which resulted in a daily intake of 5.06 mg of vitamin B_6 per day for 6 days. Supplementation at this level increased the mean E-PAL concentration significantly from 29.9 ng/ml to 39.2 ng/ml of packed cells ($P < 0.025$). The resulting mean E-PAL concentration was 136% of the undepleted mean concentration, and was significantly different ($P < 0.025$). At this daily intake of 5.06 mg of vitamin B_6 for 6 days, E-PAL concentrations in all 8 subjects were completely restored to pre-depletion levels.

In the final repletion phase of the study conducted by Fu (43), a mean daily ad libitum intake of 1.62 mg of vitamin B_6 for 11 days resulted in a mean concentration of E-PAL which was 135 % of the initial value. Thus, a comparatively higher level of PIN-HCl was required in the present study to increase E-PAL concentrations to the same degree as that required for normal women. Brown et al. (27) found that supplementation of both OC users and controls with 0.8 mg of PIN-HCl did not restore plasma PAL to starting values, but 2.0 mg or 20.0 mg supplements elevated these levels above normal following consumption of a diet containing about 0.2 mg of vitamin B_6/day for 1 menstrual cycle. No appreciable differences in plasma PAL were seen between OC users and controls during repletion.

The effect of the supplementation of varying levels of PIN-HCl for varying lengths of time on the level of PAL in the erythrocyte in the OC users of the present study and in the nonusers of the study by Fu (43) is shown in Table 11. The response to supplementation was much smaller in the users of OC than that found in nonusers of OC. Although phase 1 of repletion in both studies was identical in level of supplementation

Table 11. Response of E-PAL to various levels of supplementation with PIN-HCl in users and nonusers of OC after 32 and 43 days of depletion, respectively.

		E-PAL[a]	A Vitamin B_6 Intake[b]	B Days of Repletion[c]	C % Increase[d]	C+B % Increase per day	$\frac{C}{AxB}$ % Increase per mg vitamin B_6 consumed
OC Users	Depleted	14.0	0.36				
	Repletion 1	21.2	0.96	7	51.4	7.3	7.6
	Repletion 2	29.9	1.56	8	41.0	5.1	3.3
	Repletion 3	39.2	5.06	6	31.1	5.2	1.0
	Total[e]		49.56	21			
Non-Users (43)	Depleted	46	0.34				
	Repletion 1	188	0.96	7	308.7	44.1	45.9
	Repletion 2	263	1.56	3	39.9	13,3	8.5
	Repletion 3	454	1.62	11	72.6	6.6	4.1
	Total[e]		29.22	21			

a Expressed as ng/ml of packed cells.

b Expressed as mg/day.

c Indicates the number of days in each repletion period.

d Indicates percent increase in E-PAL during each repletion phase.

e Represents the total vitamin B_6 intake and total days of repletion.

and duration, the OC users showed a smaller percent increase in E-PAL concentration per mg of the vitamin consumed than the nonusers of OC. The second repletion phase was of longer duration in the study on OC users, but again a smaller percent increase in E-PAL per mg of vitamin B_6 consumed was observed. In the final repletion phase, nearly 2 times the amount of vitamin B_6 was consumed by the OC users than by the nonusers (30.36 mg vs. 17.82 mg), yet the nonusers showed a percent increase in E-PAL 4 times greater than that shown by the OC users.

In comparing the total response of E-PAL to supplemented vitamin B_6 over the entire repletion period (21 days in both studies), although the total ingestion of vitamin B_6 by users of OC was higher than that ingested by nonusers the E-PAL concentrations in OC users responded with less sensitivity. Thus, a total intake of approximately 50 mg of vitamin B_6 by OC users resulted in an increase in E-PAL from 14.0 to 39.2 ng/ml of packed cells, a total percent increase of 180% over the mean depleted value; while an intake of approximately 30 mg of the vitamin resulted in an increase in E-PAL levels from the mean depleted value of 46 ng to 454 ng/ml of packed cells in the nonusers of OC. This was an increase of 887% over the mean depleted value. Further comparison of the percent increase per mg of the vitamin consumed (calculated as total percent increase ÷ total vitamin B_6 intake) produced values of 30.4 and 3.6 for nonusers and OC users, respectively, thus illustrating that nonusers were capable of a 10 fold greater percent increase per mg of vitamin B_6 consumed than OC users.

From these calculations, the group of young women using OC showed a relative insensitivity to supplemented vitamin B_6 when compared to the control group, an observation which could reflect an increased requirement

of vitamin B_6 by this group. Based on the criterion of complete restoration of E-PAL to starting values after depletion of erythrocyte stores of vitamin B_6, this requirement was between 1.5 and 5 mg of vitamin B_6/day, as opposed to the requirement of 1.5 mg/day for normal young women (43).

Possible explanations for the small response of the erythrocyte in OC users to ingested vitamin B_6 could be that depletion of other body tissues is more severe than in the erythrocyte, and thus a longer time at a higher level of vitamin B_6 is necessary for repletion; or, that the demand for vitamin B_6 might be higher in other tissues, such as the brain or liver, thus shunting a greater amount of the vitamin away from the erythrocyte. Such a redistribution of the coenzyme may be the result of increased activity of PAL-P-dependent enzymes. It has been established that estrogen containing OC administered to female rats (38) results in increased activity of tryptophan oxygenase. The enzyme in turn stimulates the tryptophan to niacin pathway, thus increasing the need for vitamin B_6, the cofactor necessary for the several vitamin B_6 dependent interconversions.

Control Study

A group of 8 adult women not using OC consumed the low vitamin B_6 diet plus a supplement of 1.7 mg PIN-HCl/day for a 9 day period, corresponding to the adjustment period of the experimental study. Concentrations of E-PAL for the control group are shown in Table 12.

Mean E-PAL after 1 day was 24.0 ng/ml of packed cells; after 9 days the mean E-PAL level rose to 32.2 ng/ml of packed cells. The difference between these two means was significant at a $P < 0.05$.

The mean initial value for the control group was significantly lower than that for the experimental group ($P < 0.025$). This result was un-

Table 12. Effect of a constant intake of vitamin B_6[a] on mean concentration of erythrocyte pyridoxal[b] in 8 adult women not using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[a]	SUBJECTS								Mean ±SEM[c]
		I	J	K	L	M	N	O	P	
1	2.06	25.2	21.8	27.6	32.3	21.1	20.0	19.3	24.9	24.0±1.6
9	2.06	44.1	-	25.1	23.9	23.9	33.7	33.4	41.0	32.2±3.1

a 2.06 mg vitamin B_6 was consumed daily for 9 days.

b Expressed as ng/ml packed RBC.

c Standard error of the mean.

expected. It may however, reflect a difference in dietary intake of vitamin B_6 between the two groups. Without actual data describing the dietary intake of the 2 groups previous to the study, it is only speculation that the nonusers had lower intake of vitamin B_6 and thus lower E-PAL levels.

One of the major metabolic end products of vitamin B_6 metabolism is 4-pyridoxic acid (12) thus determination of this metabolite should theoretically reflect dietary intake of this vitamin. Comparing 4-PDA excreted by the 2 groups, the OC users excreted 3.42 umoles of 4-PDA/g creatinine/24 hours, which was significantly more than the 2.58 umoles/g creatinine/24 hours excreted by the nonusers ($P < 0.01$)[a]. These findings could support the supposition of a higher intake of the vitamin by the OC users.

Free vitamin B_6, mainly PAL, is excreted in the urine, thus this parameter can also be used as a reflection of recent dietary intake of vitamin B_6 (12). Analysis of urinary excretion of vitamin B_6 in the 2 groups of the present study showed no significant differences between the groups. Mean urinary vitamin B_6 level in the OC group was 74.23 ng B_6/24 hours, and although higher than the nonuser group, was not significantly different from the 60.66 ng B_6/24 hours excreted by the nonusers. Thus, the hypothesis that dietary intake of vitamin B_6 by the OC users was greater than that for the group of nonusers is inconclusive.

After consuming the adjustment diet, containing 2.06 mg vitamin B_6/day, both the OC users and nonusers stabilized at the similar E-PAL concentrations of 28.7 and 32.2 ng/ml of packed cells, respectively.

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a Unpublished data.

On the basis of the above results, there appeared to be no evidence of reduced E-PAL concentrations in the OC group while consuming 2.06 mg of vitamin B_6. Recent work by Lumeng et al. (29) has suggested that there is an age-related variation in plasma PAL-P in users and non-users of OC. He suggested that whereas E-PAL levels in OC users of 25 years or more in age were significantly reduced, those of 20 to 24 years showed no change in E-PAL. Results of the present study, therefore may be a reflection of this age-relationship in that no evidence of lowered E-PAL levels were found in OC users with a mean age of 20 years.

C. Erythrocyte Alanine Aminotransferase

E-Ala-AT Activity

The E-Ala-AT activity, in vitro PAL-P stimulated activity and in vitro PAL-P percent stimulation of E-Ala-AT in 8 female subjects using OC are presented in Tables 13, 14 and 15. E-Ala-AT activity and percent stimulation by in vitro PAL-P are illustrated in Figure 3.

Adjustment Period: During the adjustment period, 2.06 mg of vitamin B_6 was consumed daily for 10 days. The mean E-Ala-AT activity after 1 day on the study was 143.1 µg of pyruvate/ml of packed cells/hr. After 10 days, mean E-Ala-AT activity showed a nonsignificant decrease to 138.0 µg of pyruvate/ml of packed cells /hr.

Depletion Period: After 7 days on the low vitamin B_6 diet (0.36 mg/day) the mean E-Ala-AT activity of the 8 subjects rose, again nonsignificantly to 143.5 µg of pyruvate/ml of packed cells /hr. A concomitant increase was observed in E-PAL, providing further indication of a shift of the coenzyme to the erythrocyte in an initial response to a dietary deficiency of vitamin B_6.

After 14 days on the deficient diet mean E-Ala-AT activity of the subjects dropped to 102.3 µg of pyruvate/ml of packed cells/hr. This decrease resulted in a significant change from the pre-depletion level of 138.0 µg of pyruvate/ml of packed cells/hr ($P < 0.05$).

Following 21 days on the low vitamin B_6 diet a more statistically significant decrease occurred ($P < 0.025$) as mean E-Ala-AT decreased to 86.3 µg of pyruvate/ml of packed cells/hr; or to 62% of the mean undepleted activity. Depletion of the subjects continued 11 days longer and was accompanied by progressively diminishing enzyme activity. Mean E-Ala-AT activity after 28 and 32 days of depletion was 76.0 and 62.7 µg of pyruvate

Table 13. Effect of vitamin B_6 intake on E-Ala-AT activity[a] of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	225.3	93.7	56.5	199.2	167.8	221.2	88.4	93.0	143.1±23.9
10	2.06	188.1	60.0	105.4	136.4	187.6	205.4	97.8	123.0	138.0±18.2
17	0.36	211.6	67.9	111.6	162.6	188.6	191.2	94.8	120.4	143.6±18.4
24	0.36	164.4	60.0	47.6	153.0	152.3	99.2	86.1	55.8	102.3±17.0
31	0.36	129.2	55.1	48.9	-	107.8	92.5	84.1	-	86.3±10.4
38	0.36	111.1	47.2	60.0	133.9	83.7	52.4	62.4	57.2	76.0±11.0
42	0.36	104.7	16.2	35.8	93.0	92.0	56.9	49.3	45.8	61.7±11.1
49	0.96[d]	106.5	28.9	57.2	94.1	55.0	58.9	44.8	75.5	65.1± 9.0
58	1.56[e]	155.7	45.1	-	136.8	130.9	96.8	81.0	95.1	105.9±14.3
65	5.06[f]	201.6	59.3	78.6	196.4	136.4	129.7	115.8	119.2	129.6±17.7

a Expressed as μg pyruvate/ml packed RBC/hr.

b Expressed as mg/day.

c Standard error of the mean.

d 0.6 mg of PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg of PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg of PIN-HCl was supplemented daily between day 60 and 66.

Table 14. Effect of vitamin B_6 intake on in vitro stimulation of E-Ala-AT[a] with PAL-P of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	227.9	101.0	66.2	207.2	180.6	242.6	114.1	125.8	158.2±23.0
10	2.06	212.6	-	105.4	-	196.1	220.9	112.7	139.6	164.5±21.0
17	0.36	233.6	77.5	134.0	168.5	210.4	216.4	94.1	145.1	159.9±20.4
24	0.36	161.9	65.6	71.3	156.4	172.1	110.6	103.4	74.9	114.5±15.4
31	0.36	140.6	70.6	55.8	-	126.4	131.6	88.9	-	102.3±14.4
38	0.36	122.5	57.2	-	152.5	86.3	70.3	77.2	59.4	89.3±13.4
42	0.36	128.2	26.4	42.4	106.8	107.5	64.6	62.7	55.3	74.2±12.6
49	0.96[d]	128.7	38.2	73.7	114.4	-	69.9	52.0	107.5	83.5±12.8
58	1.56[e]	166.4	53.8	-	138.5	150.4	107.5	101.3	114.7	119.0±14.1
65	5.06[f]	215.0	71.0	80.6	203.1	154.4	146.3	135.4	132.3	142.3±18.0

a Expressed as µg pyruvate/ml packed RBC/hr.

b Expressed as mg/day.

c Standard error of the mean.

d 0.6 mg of PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg of PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg of PIN-HCl was supplemented daily between day 60 and 66.

Table 15. Effect of vitamin B_6 intake on percent in vitro PAL-P stimulation[a] of E-Ala-AT of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								MEAN ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	1.2	7.8	17.2	4.0	7.6	9.7	29.0	35.3	14.0±4.3
10	2.06	13.0	-	0	-	4.5	7.5	15.2	13.5	9.0±2.5
17	0.36	10.4	14.1	20.1	3.6	11.6	13.2	0	20.5	11.7±2.5
24	0.36	0	9.3	49.8	2.2	13.0	11.5	20.1	34.2	17.5±6.0
31	0.36	8.8	28.1	14.1	-	17.2	42.3	5.7	-	19.4±5.6
38	0.36	10.3	21.2	-	13.9	3.1	34.2	23.7	3.8	15.7±4.3
42	0.36	22.4	63.0	18.4	14.8	16.8	13.5	27.2	20.7	24.6±5.7
49	0.96[d]	20.8	32.2	28.8	21.6	-	18.7	16.1	42.4	25.8±3.5
58	1.56[e]	6.9	19.3	-	1.2	14.9	11.1	25.1	20.6	14.2±3.2
65	2.06[f]	6.6	19.7	2.5	3.4	13.2	12.8	16.9	11.0	10.8±2.2

a Percent stimulation = $\frac{\text{E-Ala-AT activity of in vitro PAL-P stimulation - E-Ala-AT Activity}}{\text{E-Ala-AT activity}} \times 100$

b Expressed as mg/day.

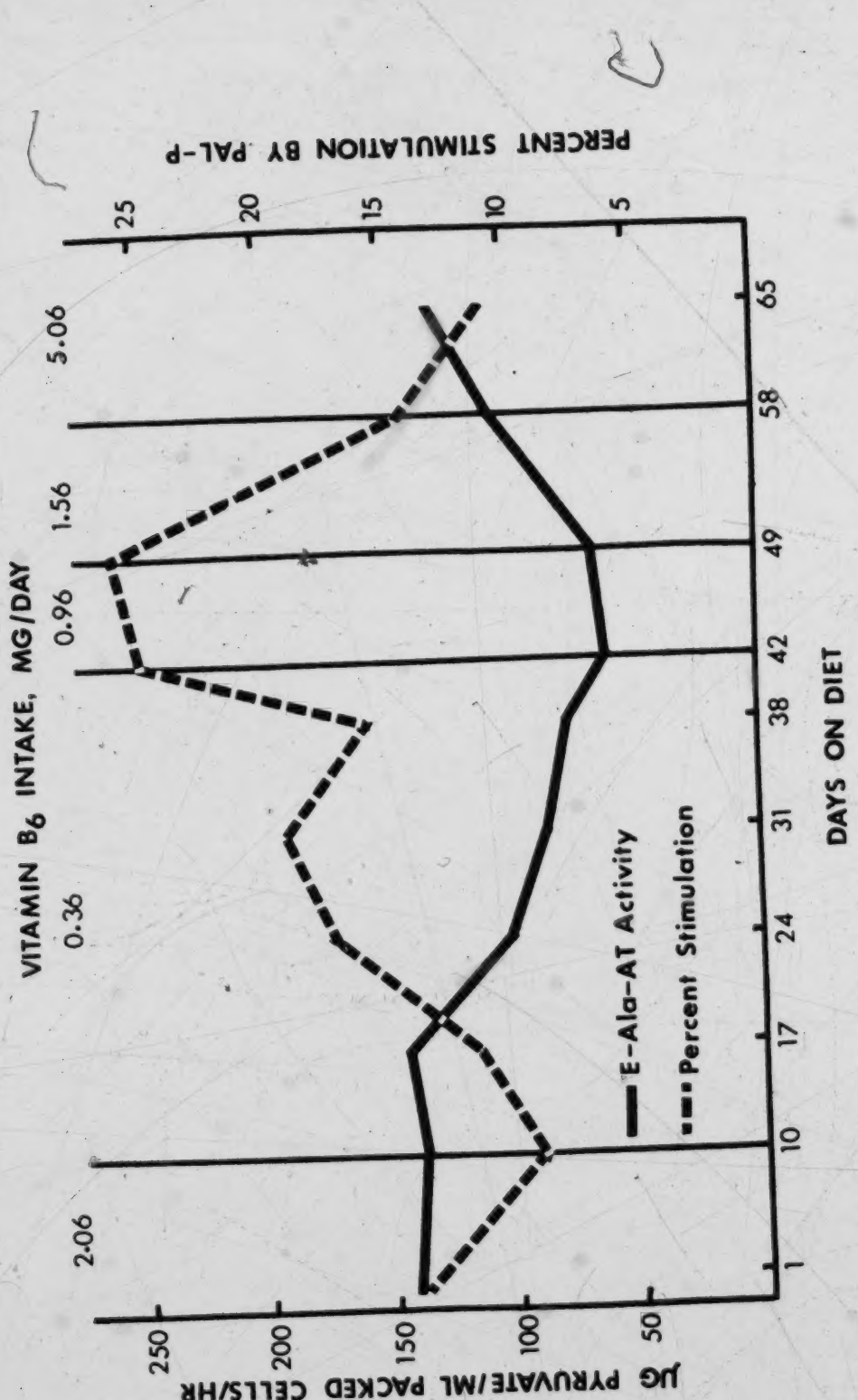
c Standard error of the mean.

d 0.6 mg of PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg of PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg of PIN-HCl was supplemented daily between day 60 and 66.

Figure 3. Effect of vitamin B_6 intake on E-Ala-AT activity and percent stimulation with in vitro PAL-P of 8 adult women using oral contraceptives.


PERCENT STIMULATION BY PAL-P
25
20
15
10
5
5.06
1.56
0.96
0.36
VITAMIN B6 INTAKE, MG/DAY
2.06
μG PYRUVATE/ML PACKED CELLS/HR
250
200
150
100
50
1
10
17
24
31
38
42
49
58
65
DAYS ON DIET
E-Ala-AT Activity
Percent Stimulation

/ml of packed cells/hr, respectively. E-Ala-AT activity at the height of depletion was only 45% that of the mean undepleted value. The difference between the two levels was highly significant ($P < 0.001$). The decreasing E-Ala-AT activity was accompanied by a reduction in E-PAL concentration.

Raica and Sauberlich (30) detected only small amounts of E-Ala-AT activity in adult men, unresponsive to either depletion or supplementation with vitamin B_6. Fu (43) obtained similar results in a group of adult women undergoing vitamin B_6 depletion and repletion. Baysal et al. (22) and Cinnamon and Beaton (31) however, have shown E-Ala-AT activity in adult men fed low vitamin B_6 diets to decline to levels significantly lower ($P < 0.01$) than predepletion levels. Rose et al. (18) found E-Ala-AT activity to decrease to a level 58% of the starting value in one male subject consuming a low vitamin B_6 diet for 20 days.

The discrepancy in the above findings may be due to a difference in methodology, as both Raica and Sauberlich (30) and Fu (43) used a spectrophotometric method whereas results reported by Baysal et al. (22), Cinnamon and Beaton (31), and in the present study were obtained by a colorimetric method.

Repletion Period: A daily supplement of 0.6 mg of PIN-HCl for 7 days resulted in increasing E-Ala-AT activity in 6 of the 8 subjects. Subjects E and G did not respond to the supplement; E-Ala-AT in these subjects actually declined slightly. Mean E-Ala-AT activity increased nonsignificantly from 61.7 to 65.0 μg of pyruvate/ml of packed cells/hr, or to 47% of the initial activity.

The consumption of a daily supplement of 1.2 mg of PIN-HCl for the next 8 days produced substantial increases in E-Ala-AT activities in all

subjects. The mean activity (105 μg of pyruvate/ml of packed cells/hr) resulted in an increase in E-Ala-AT from 47% to 77% of the initial level. This increase was statistically significant ($P < 0.05$). Although none of the individual values reached their respective initial values at the above level of supplementation, the mean value was not significantly different from the mean undepleted value.

The final phase of repletion, consisting of a daily supplement of 4.7 mg of PIN-HCl for 6 days, resulted in a continual increase in the mean E-Ala-AT activity of the subjects, from 105.9 to 129.6 μg of pyruvate /ml of packed cells/hr. Mean E-Ala-AT activity was increased to 94% of the mean initial activity, with individual values restored to original levels in 4 of the 8 subjects. Although the mean E-Ala-AT activity on the last day of the study was still below the pre-depletion value, there was no significant difference between the two means.

In the study by Baysal (22), subjects consumed a diet containing 0.16 mg of vitamin B_6 per day for 27 to 40 days and were then supplemented with 0.6 or 0.9 mg of PIN-HCl for 10 or 20 days. Only slight increases in E-Ala-AT activity were observed. Further supplementation with 50 mg of PIN-HCl daily for 3 days did not restore E-Ala-AT activity to pre-depletion levels. Cinnamon and Beaton (31) reported that E-Ala-AT in their vitamin B_6 depleted subjects did not return to normal until after 3 to 4 weeks of daily supplementation with 2.0 mg of PIN-HCl. Rose (18), however, using 20 mg of PIN-HCl/day for 9 days was able to demonstrate complete restoration of E-Ala-AT activity in his subject.

The general consensus is that E-Ala-AT is more severely affected than other aminotransferases and restoration of this enzyme system occurs with difficulty. These findings are substantiated by studies in the rat

(69, 70). Results from the present study tend to indicate that like normal adults, E-Ala-AT levels in women using OC decrease sensitively to decreased dietary intakes of vitamin B_6 and respond slowly to in vivo supplementation of the vitamin. The OC users, however, required vitamin B_6 in amounts in excess of the normal adult women to restore E-Ala-AT activity to the pre-depletion level. The effect of vitamin B_6 supplementation on restoration of E-Ala-AT activity was parallel to that observed in restoration of E-PAL levels: both systems responded slowly and no effect could be attributed to the type of OC preparation used or the duration of its use.

The slow restoration of E-Ala-AT activity to pre-depletion levels by in vivo PIN-HCl suggests that the decreased activity produced by vitamin B_6 deprivation might be due to apoenzyme depletion, since it is known that apoenzyme synthesis is vitamin B_6 dependent (41). If a deficiency of apoenzyme did exist, addition of the coenzyme in vitro in the form of pyridoxal phosphate would produce little if any additional enzymatic activity. In the present study, however, addition of 50 µg of PAL-P to an in vitro system resulted in an increasing percent stimulation as the deficiency progressed, indicating that the decreasing E-Ala-AT activity was due presumably to deficiency of the coenzyme only.

An alternate explanation for the lack of response of E-Ala-AT activity during repletion might be that a redistribution of the coenzyme to some other enzyme system had occurred, leaving insufficient amounts for the E-Ala-AT system. It has been suggested by Brin et al. (70) that Asp-AT apoenzyme in rat tissues and plasma has a greater affinity for vitamin B_6 than does Ala-AT. Cavill et al. (71) found that human E-Asp-AT combines more strongly with the coenzyme while E-Ala-AT appeared to be more sensitive to vitamin B_6 deficiency. Erythrocytes from subjects

studied by Cinnamon and Beaton (31) also appeared to contain Asp-AT possessing a greater affinity for the coenzyme than did Ala-AT.

Fu (43) suggested that a mechanism for the slow response of the aminotransferase to supplementation might be due to the slow turnover rate of the erythrocyte in the human. The duration of the repletion period may not have been of sufficient length for enough newly synthesized cells containing the increased levels of vitamin B_6 to accumulate. The use of OC, superimposed on all these mechanisms, may further impede the rapid and complete restoration of E-Ala-AT activity as estrogens may cause a further redistribution of the vitamin between apoenzymes (72).

Percent stimulation of E-Ala-AT with in vitro PAL-P

Adjustment Period: The mean percent stimulation of E-Ala-AT by in vitro PAL-P in 8 subjects using anovulatory steroids after 1 day on the adjustment diet was 14.0%. After 10 days on the same diet, mean percent stimulation of E-Ala-AT dropped to 9.0%. This mean was derived from 6 subjects because of insufficient sample from subjects B and D.

Woodring and Storvick (49) reported that with normal individuals there is little stimulation of E-Ala-AT. Percent stimulation in 45 normal subjects ranged from 0% to 5%. Cheney et al. (51) and Sauberlich et al. (12) reported that stimulation with in vitro PAL-P seldom exceeded 25%. The same appears to be true for women using OC. Rose (18, 33) in two separate studies reported mean stimulation of E-Ala-AT with in vitro PAL-P to be 20% and 25% in the 2 groups of OC users studied. The initial values reported for the OC users of the present study are in a similar range as reported by others for normal subjects.

Depletion Period: As the depletion period progressed the trend observed

in mean in vitro percent stimulation of E-Ala-AT was one of gradual increase. Mean values for percent stimulation for the first 3 weeks of depletion increased from the undepleted value of 9.0% to 11.7%, 17.5% and 19.4% after 17, 24, and 31 days on the study, respectively. These increases were not statistically significant. An unexpected drop in percent stimulation, inconsistent with the corresponding basal E-Ala-AT activity, occurred after 38 days on the study. It too was not statistically significant. Mean percent stimulation at the height of depletion however, increased to 24.6% and was once more consistent with the preceding trend of increasing percent stimulation of E-Ala-AT. This increase would indicate that although erythrocyte levels of the vitamin B_6 coenzyme were diminishing, as evidenced by decreasing basal activity of E-Ala-AT, the level of apoenzyme remained constant.

Repletion Period: After 7 days of supplementation with 0.6 mg of PIN-HCl per day, mean in vitro percent PAL-P stimulation of E-Ala-AT rose unexpectedly to 25.8%. This value was not significantly different from the value at the height of depletion, however, this slight rise was not consistent with the increase observed in both the mean E-Ala-AT activity and E-PAL level.

Following 8 days of further supplementation with 1.2 mg of PIN-HCl /day, the mean in vitro PAL-P percent stimulation of the subjects decreased from 25.8% to 14.2%, a decrease significant at a $P < 0.05$. The difference between the mean initial value and the mean value after vitamin B_6 supplementation at the 1.56 mg level was not significantly different.

The final phase of repletion diminished still further the percent in vitro PAL-P stimulation. After 6 days of daily supplementation with 4.7 mg of PIN-HCl, percent stimulation decreased from 14.2% to 10.8%.

Although the mean initial and final values for percent stimulation were not significantly different, only 4 of 6 subjects achieved values equal to or below that of their pre-depletion levels. The data for E-Ala-AT in the present study are thus consistent with the previous finding that E-Ala-AT is sensitive to a dietary deficiency of vitamin B_6 and is slow to respond to in vivo supplementation. The data also suggest that some adult women using OC may require an amount of vitamin B_6 in excess of that required by normal adult women.

Cinnamon and Beaton (31) suggested that the measurement of in vitro E-Ala-AT stimulation with PAL-P was the most meaningful parameter in the assessment of vitamin B_6 nutriture. Although the present study does indicate that percent in vitro PAL-P stimulation of E-Ala-AT tends to reflect vitamin B_6 intake, individual values show a great deal of fluctuation. This variability may be the result of non-enzymatic transamination which is known to occur (73). Additional studies, particularly those on subjects who are using OC, are necessary to establish the validity and usefulness of this parameter.

Control Study

The E-Ala-AT activity, PAL-P stimulated activity and percent stimulation of E-Ala-AT of 8 adult women not using OC are presented in Table 16. The study period of the control group corresponded to the adjustment period of the experimental study. Mean E-Ala-AT activity after 1 day was 105.8 µg of pyruvate/ml of packed cells/hr. Although this value was lower than that obtained initially in the experimental study, the difference between the 2 means was not significant. After consuming this diet for 9 days, mean E-Ala-AT activity of the subjects diminished slightly

Table 16. Effect of a constant intake of vitamin B_6[a] on E-Ala-AT activity[b], in vitro PAL-P stimulated activity[b] and % stimulation[c] in 8 adult women not using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[a]	SUBJECTS I	J	K	L	M	N	O	P	Mean ±SEM[d]
Activity[b]										
1	2.06	61.0	106.1	136.8	141.6	96.5	76.5	126.8	101.3	105.8±10.0
9	2.06	50.0	-	165.0	95.8	94.8	64.1	160.6	95.8	104.0±16.0
Stimulated Activity[b]										
1	2.06	70.3	134.4	150.9	161.2	113.2	88.4	144.0	108.5	121.4±11.2
9	2.06	66.2	-	188.8	112.2	123.4	79.2	175.7	117.2	123.2±22.4
% Stimulation[c]										
1	2.06	15.5	26.7	10.3	13.8	17.3	15.6	13.6	7.1	15.0±2.0
9	2.06	32.4	-	14.2	17.1	30.2	23.6	9.4	22.3	21.3±3.2

a 2.06 mg of vitamin B_6 was consumed daily for 9 days.

b Expressed as ug pyruvate/ml of packed RBC/hr.

c % stimulation = $\frac{\text{in vitro PAL-P stimulated E-Ala-AT activity - E-Ala-AT activity}}{\text{E-Ala-AT activity}} \times 100$

d Standard error of the mean.

to 104.0 μg of pyruvate/ml of packed cells/hr. There was no significant difference between this and the corresponding value obtained from the experimental subjects.

The mean in vitro PAL-P percent stimulation after 1 and 9 days on the control study was 15.0% and 21.3%, respectively. When compared to the experimental values, there was no significant difference between initial values, however, the final value of the control study was significantly higher than the corresponding experimental study value ($P < 0.025$). This result was unexpected.

The effect of OC usage on E-Ala-AT activity and percent stimulation has been investigated by many researchers. A comparison of some of their findings appears in Table 17. Aly et al. (16) in a comparison of E-Ala-AT levels in users and nonusers of OC found no significant difference in the 2 groups. In vitro PAL-P stimulation was not investigated.

Rose et al. (18) also observed no significant differences between either basal E-Ala-AT activity or the PAL-P stimulation in a group of nonusers of OC and a group of OC users who had used the preparations for 6 to 36 months. Doberenz et al. (32) measured E-Ala-AT activity and percent in vitro stimulation by PAL-P in 11 nonusers and 13 OC users. In women using OC for an average length of 2.5 years, mean basal E-Ala-AT activity was significantly lower ($P < 0.02$) and percent stimulation by PAL-P was significantly greater ($P < 0.001$) than that of nonusers. Rose et al. (33) found a significant increase in the in vitro PAL-P stimulation of E-Ala-AT in a group of 80 OC steroid-treated women as compared to a group of 50 controls ($P < 0.025$). No difference was observed in the basal activities of this enzyme. Thus, it is evident that OC therapy has produced inconsistent effects on E-Ala-AT activity and in vitro PAL-P

Table 17. Comparison of mean E-Ala-AT activity and percent stimulation by in vitro PAL-P in nonusers and users of oral contraceptives.

Investigator	E-Ala-AT Activity[a]		E-Ala-AT Stimulation (%)	
Present study				
nonusers	104	NS[d]	21	P < 0.025
OC users	138		9	
Aly et al. (16)[b]				
nonusers	546	NS	-	
OC users	546			
Rose et al. (18)[c]				
nonusers	290	NS	17	NS
OC users	307		20	
Doberenz et al. (32)				
nonusers	177	P < 0.02	22	P < 0.001
OC users	90		118	
Rose et al. (33)[c]				
nonusers	300	NS	18	P < 0.025
OC users	343		25	

a Activity is expressed in µg pyruvate/ml of packed RBC/hr.

b Calculated values. Conversion based on 1 mole pyruvate = 88 g.

c Calculated values. Conversion based on normal blood values for the female: Hct - 42%, Hgb - 14 g/100 ml blood.

d Not statistically significant.

stimulation when compared to normal women.

The measurement of E-Ala-AT activity and percent stimulation with in vitro PAL-P in the OC users of the present study while consuming 2.06 mg of vitamin B_6 daily did not indicate a vitamin B_6 deficient state. The data, however, would tend to suggest that this group, after the stress of a dietary deficiency of vitamin B_6, required higher levels of the vitamin to restore aminotransferase activity to pre-depletion levels than is agreed upon as the recommended daily allowance for normal adult women (11). The amount of the vitamin that was required was between 1.5 and 5 mg per day and is similar to the amount of vitamin B_6 required to restore E-PAL to levels observed before depletion.

D. Erythrocyte Aspartate Aminotransferase

The E-Asp-AT activity, in vitro PAL-P stimulated activity and percent stimulation of E-Asp-AT by PAL-P in 8 female subjects using OC is presented in Tables 18, 19 and 20. E-Asp-AT activity and in vitro PAL-P percent stimulation are illustrated in Figure 4.

E-Asp-AT activity

Mean E-Asp-AT activity ranged from 38.1 to 82.0 μmoles NAD/ml packed cells/hr during the entire study. These values are somewhat higher than those reported by Fu (43) where E-Asp-AT activity ranged from 21.7 to 61.0 μmoles NAD/ml of packed cells/hr in 8 nonusers of OC consuming a diet similar to the one in the present study. Unlike Fu's results for normal women, E-Asp-AT in OC users of the present study showed no systematic response to deprivation of dietary vitamin B_6. Throughout the entire depletion phase weekly, random fluctuations in E-Asp-AT activity, unrelated to dietary intake of vitamin B_6 and inconsistent with trends observed in E-PAL levels or E-Ala-AT activity, were observed. No changes in E-Asp-AT activity were significant throughout the depletion phase. Similar fluctuation in E-Asp-AT activity was reported by Rose et al. (18).

After 32 days of depletion, the mean E-Asp-AT activity diminished to a level significantly lower than that observed initially ($P < 0.001$). All subjects showed a decrease at the end of depletion, but whether E-Asp-AT would have continued to decline had the depletion phase been extended is unknown. Possibly the E-Asp-AT system in women using OC requires a longer time to become deficient in coenzyme than was allowed in the present study.

Observations in the rat deprived of dietary vitamin B_6 indicated that liver Asp-AT is insensitive to vitamin B_6 deficiency (69, 70). This

Table 18. Effect of vitamin B_6 intake on E-Asp-AT activity [a] of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	58.3	49.2	52.9	65.4	66.4	69.2	44.3	68.6	59.3±3.4
10	2.06	75.3	50.7	59.4	80.5	66.6	79.1	45.8	72.0	66.2±4.6
17	0.36	67.0	52.0	55.6	76.6	56.3	79.1	39.4	68.0	61.8±4.7
24	0.36	70.9	55.0	46.1	73.0	74.6	77.5	54.9	76.7	66.1±4.3
31	0.36	63.9	49.7	48.3	72.2	61.8	64.9	48.8	-	58.5±3.4
38	0.36	70.6	48.6	62.7	82.0	60.9	70.3	47.9	70.9	64.2±4.1
42	0.36	56.8	41.6	48.2	65.4	52.3	63.0	41.2	55.0	52.9±3.2
49	0.96[d]	53.2	46.6	50.9	62.7	52.4	51.8	44.0	63.9	53.2±2.5
58	1.56[e]	60.1	38.1	51.0	67.0	54.1	54.5	47.4	56.8	53.6±3.0
65	5.06[f]	68.5	48.0	48.8	81.0	57.8	61.1	51.9	63.7	60.1±3.9

a Expressed as μmoles NAD/ml of packed RBC/hr.

b Expressed as mg/day.

c Standard error of the mean.

d 0.6 mg of PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg of PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg of PIN-HCl was supplemented daily between day 60 and 66.

Table 19. Effect of vitamin B_6 intake on in vitro stimulation of E-Asp-AT[a] with PAL-P of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	115.0	93.2	115.0	119.2	107.8	108.1	85.6	137.2	110.0±5.6
10	2.06	137.6	98.4	115.2	131.4	124.8	133.1	87.4	143.0	121.4±6.9
17	0.36	117.4	111.6	117.8	145.5	124.6	134.9	92.4	131.2	121.9±5.7
24	0.36	132.2	106.9	102.1	123.7	108.1	129.4	100.3	154.6	119.7±6.6
31	0.36	130.6	94.9	110.6	131.0	124.0	120.9	90.4	-	114.6±6.2
38	0.36	131.2	97.8	127.1	149.1	110.0	125.2	102.0	132.2	121.9±6.1
42	0.36	108.0	94.4	101.3	120.7	100.9	114.8	81.7	113.7	104.4±4.4
49	0.96[d]	104.4	93.8	104.0	106.0	98.2	96.9	89.9	132.6	103.2±4.6
58	1.56[e]	109.4	83.0	107.0	109.6	97.2	103.5	91.3	120.9	102.7±4.2
65	5.06[f]	120.5	98.1	103.5	138.9	102.6	108.0	108.8	128.3	113.6±5.0

a Expressed as μmoles NAD/ml of packed RBC/hr.

b Expressed as mg/day.

c Standard error of the mean.

d 0.6 mg of PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg of PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg of PIN-HCl was supplemented daily between day 60 and 66.

Table 20. Effect of vitamin B_6 intake on percent in vitro PAL-P stimulation of E-Asp-AT[a] of 8 adult women using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[b]	SUBJECTS								Mean ±SEM[c]
		A	B	C	D	E	F	G	H	
1	2.06	97.3	89.2	117.5	82.2	62.2	56.3	93.0	100.2	87.2±7.1
10	2.06	82.6	93.8	94.1	63.2	87.4	68.3	91.0	98.7	84.9±4.5
17	0.36	75.2	114.7	111.6	89.8	121.1	70.6	134.8	92.8	101.3±8.0
24	0.36	86.5	94.4	121.4	69.4	45.0	66.9	82.6	101.5	83.5±8.3
31	0.36	104.5	91.2	128.9	81.5	100.7	86.2	85.5	-	96.9±5.8
38	0.36	85.9	101.1	102.6	81.8	81.3	78.2	112.8	86.5	91.3±4.4
42	0.36	90.2	126.8	110.3	84.4	92.7	82.2	98.2	106.8	99.0±5.3
49	0.96[d]	96.2	101.2	104.3	69.0	87.4	87.2	104.3	107.5	94.6±4.6
58	1.56[e]	82.0	117.8	110.0	63.6	79.5	89.7	92.7	112.7	93.5±6.6
65	5.06[f]	75.9	104.5	112.0	71.5	77.7	76.9	109.6	101.4	91.2±6.1

a % stimulation = $\frac{\text{in vitro PAL-P stimulated E-Asp-AT activity - E-AspAT activity}}{\text{E-Asp-AT activity}} \times 100$

b Expressed as mg/day.

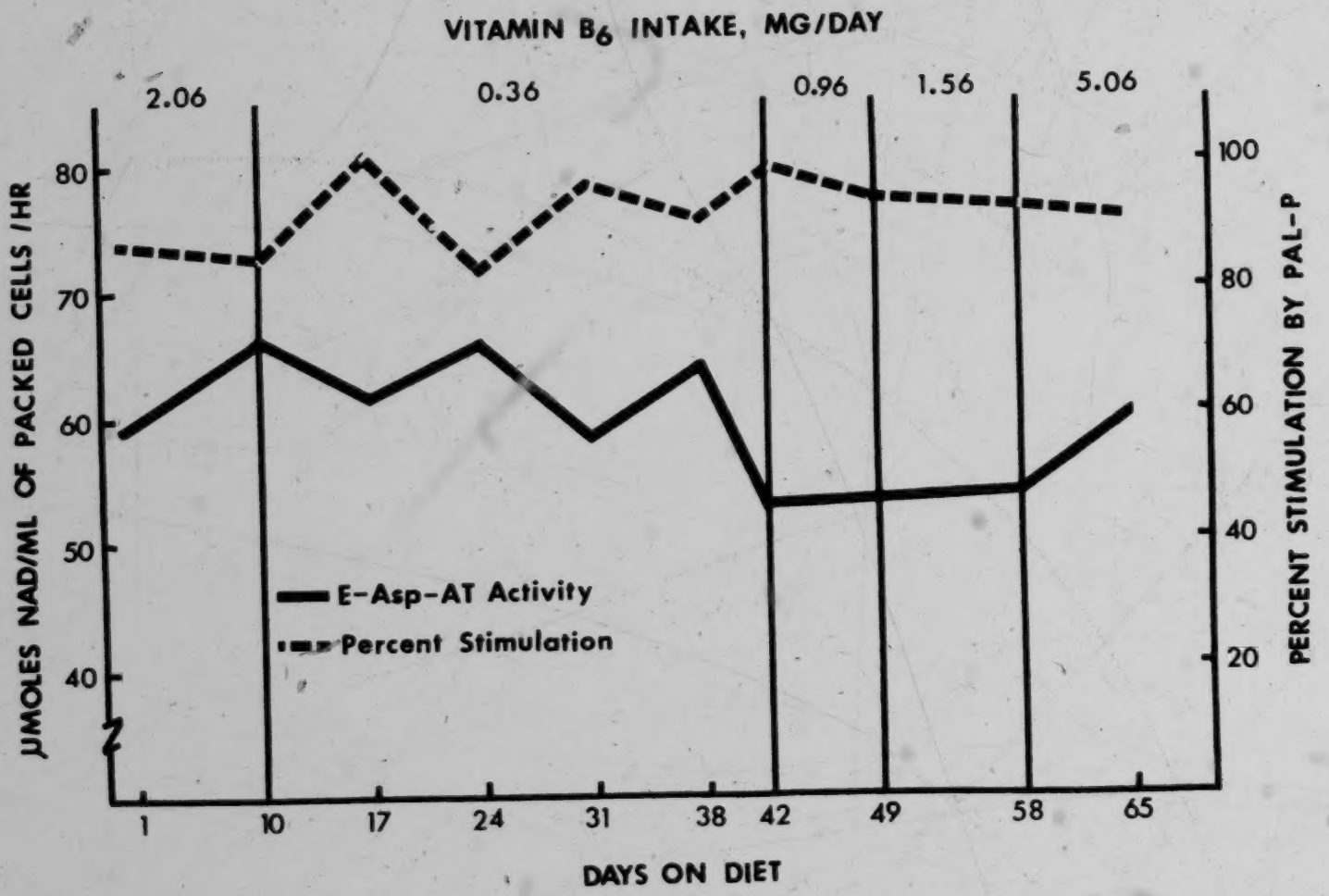
c Standard error of the mean.

d 0.6 mg of PIN-HCl was supplemented daily between day 43 and 50.

e 1.2 mg of PIN-HCl was supplemented daily between day 51 and 59.

f 4.7 mg of PIN-HCl was supplemented daily between day 60 and 66.

Figure 4. Effect of vitamin B_6 intake on E-Asp-AT activity and percent stimulation with in vitro PAL-P of 8 adult women using oral contraceptives.


VITAMIN B6 INTAKE, MG/DAY
2.06
0.36
0.96
1.56
5.06
80
70
60
50
40
100
80
60
40
20
E-Asp-AT Activity
Percent Stimulation
1
10
17
24
31
38
42
49
58
65
DAYS ON DIET
µMOLES NAD/ML OF PACKED CELLS /HR
PERCENT STIMULATION BY PAL-P

finding has been extended to human erythrocytes (22, 71) with the conclusion that E-Asp-AT might have a greater affinity for its coenzyme - PAL-P. The observation in the present study that E-Asp-AT activity did not decrease significantly until 32 days of depletion had elapsed would also lend support to this conclusion.

Response of the E-Asp-AT system to supplemented PIN-HCl did not consistently reflect intake of this vitamin. In phase 1 and 2 of repletion, daily intakes of 0.96 mg and 1.56 mg of vitamin B_6 for 7 and 8 days respectively resulted in no significant change in E-Asp-AT activity from that observed at the height of depletion. During the third phase of repletion (5.06 mg vitamin B_6 for 6 days) E-Asp-AT activity did increase significantly ($P < 0.01$). Although mean enzyme activity at termination of the study was only 91% of the mean undepleted activity, the difference between the two means was not significant.

Other workers have also found E-Asp-AT to be rather unresponsive to supplementation with vitamin B_6. Fu (43) found mean E-Asp-AT activity of 8 nonusers of OC who had consumed a low vitamin B_6 diet for 43 days to be restored to 64% of the undepleted activity after daily intakes of 0.94, 1.54 and 1.62 mg of the vitamin for 7, 3, and 11 days, respectively. Raica and Sauberlich (30) observed E-Asp-AT in male subjects consuming low vitamin B_6 diets of either high or low quantities of protein, and found the enzyme activity to reach only 70% of the undepleted activity on supplementation with 0.75 to 1.75 mg of vitamin B_6 daily for 12 weeks. Because of the tendency of basal E-Asp-AT to randomly fluctuate and to respond inconsistently to in vivo supplementation with vitamin B_6, it is not a reliable indicator of vitamin B_6 deficiency.

Percent stimulation of E-Asp-AT with in vitro PAL-P

The percent stimulation of E-Asp-AT produced weekly fluctuations that corresponded to the unstimulated enzyme activity. Where E-Asp-AT activity showed a decline, the percent stimulation of E-Asp-AT showed an increase (see Figure 4). There was no indication of coenzyme deficiency during the depletion phase, as would have been the case had the percent stimulation of E-Asp-AT continued to increase.

Supplementation with the varying levels of PIN-HCl produced a response in percent stimulation of E-Asp-AT very similar to that produced in the basal E-Asp-AT activity. Both basal and stimulated values were slow in returning to initial levels. Even after the final phase of repletion, only 2 of the 8 subjects achieved percent stimulations equal to or below the initial level.

Although other workers (30, 31) have found percent stimulation of E-Asp-AT with in vitro PAL-P to provide an acceptable indicator of vitamin B_6 status, this did not appear to be the case for the OC users of the present study. No consistency in response was observed as a result of the type of OC used or the duration of its use.

Control Study

The E-Asp-AT activities, PAL-P stimulated activities and percent stimulations of E-Asp-AT with in vitro PAL-P of 8 nonusers of OC are presented in Table 21.

Mean E-Asp-AT activity of this group of nonusers after 1 day on the control study was 56.8 μmoles/ml of packed cells /hr; a value very similar to the mean E-Asp-AT activity of the OC users shown on the same day. After consuming the adjustment diet, containing 2.06 mg of vitamin B_6

Table 21. Effect of a constant intake of vitamin B_6[a] on E-Asp-AT activity[b], in vitro PAL-P stimulated activity[b] and percent stimulation[c] in 8 adult women not using oral contraceptives.

Days on Diet	Vitamin B_6 Intake[a]	SUBJECTS I	J	K	L	M	N	O	P	Mean ±SEM[d]
Activity[b]										
1	2.06	58.6	54.5	64.1	69.3	50.0	50.8	48.2	58.6	58.6± 2.6
9	2.06	47.8	-	61.6	63.1	32.5	45.6	38.3	49.6	48.4± 4.2
Stimulated Activity[b]										
1	2.06	124.3	110.8	124.3	141.5	92.4	100.8	92.7	90.4	109.6± 6.6
9	2.06	130.0	-	103.1	114.5	67.2	88.6	81.1	100.6	97.9± 7.9
% Stimulation[c]										
1	2.06	112.1	103.4	94.1	104.2	85.0	98.4	92.3	54.3	93.0± 6.3
9	2.06	172.1	-	67.3	81.4	107.0	94.2	111.8	102.9	105.2±12.6

a 2.06 mg of vitamin B_6 was consumed daily for 9 days.

b Expressed as μmoles NAD/ml of packed RBC/hr.

c % stimulation = $\frac{\text{in vitro PAL-P stimulated E-Asp-AT activity - E-Asp-AT activity}}{\text{E-Asp-AT activity}} \times 100$

d Standard error of the mean.

per day for 9 days, mean E-Asp-AT activity of the nonusers of OC dropped to 48.4 μmoles/ml of packed cells/hr. The values after 1 and 9 days were not significantly different. The unpaired t-test indicated E-Asp-AT activity of OC users to be significantly higher ($P < 0.025$) than that of the nonusers of OC, with both groups consuming the same controlled diet for a similar period of time.

Mean in vitro percent stimulations of E-Asp-AT activities in the control group after 1 and 9 days of the control study were 93.0% and 105.2%, respectively. These values were not significantly different from each other, nor were they significantly different from the corresponding values of the group using OC.

The effect of OC usage on E-Asp-AT activity and percent stimulation has been investigated by others. A comparison of the findings of the present study with those of Aly et al. (16) and Rose et al. (18, 33) is presented in Table 22. In all studies, a significantly higher basal E-Asp-AT activity was shown in OC users than in nonusers. The trend in percent stimulation with in vitro PAL-P of the present study was similar to that in both studies by Rose (18, 33) in that somewhat higher percent stimulations were shown by nonusers of OC. It has been stated that E-Asp-AT has a great affinity for the coenzyme (71) and perhaps the affinity is even greater in women using OC. The possibility then exists, that the apoenzyme in OC users was more fully saturated with coenzyme, thus capable of lesser stimulation with PAL-P in vitro. In all four studies, however, the differences in mean percent stimulation between OC users and nonusers were not significant.

The data for basal E-Asp-AT activity indicate a definite increase in the activity of this enzyme in women taking OC. As pointed out by Aly

Table 22. Comparison of mean E-Asp-AT activity and percent stimulation by in vitro PAL-P in users and nonusers of oral contraceptives.

Investigator	E-Asp-AT Activity		E-Asp-AT Stimulation (%)	
Present study[a]				
nonusers	48.4	$P < 0.025$	105.2	NS[c]
OC users	66.2		84.9	
Aly et al. (16)[a]				
nonusers	34.3	$P < 0.01$	45.5	NS
OC users	45.9		65.1	
Rose et al. (18)[b]				
nonusers	22.0	$P < 0.01$	78.0	NS
OC users	25.9		72.0	
Rose et al. (33)[b]				
nonusers	22.5	$P < 0.05$	77.0	NS
OC users	24.6		71.0	

a Activity is expressed in μmoles NAD/ml of packed RBC/hr.

b Activity is expressed in μg oxaloacetate/mg hemoglobin/hr.

c Not statistically significant.

et al. (16) this higher E-Asp-AT activity is not in itself an indication of vitamin B_6 deficiency, when E-Asp-AT should show a decrease. Rose et al. (33) stated that assay of E-Asp-AT would be of little value for the identification of vitamin B_6 deficiency in women using OC. This higher E-Asp-AT level in OC users does suggest either a more active enzyme system or an increased synthesis of apoenzyme or both. The ultimate consequence would be an increased need for the coenzyme PAL-P. Rose et al (33) has suggested that elevated E-Asp-AT could also be due to stabilization of the enzyme molecule with a decrease in the rate of degradation.

Whether or not the consumption of estrogen containing oral contraceptives is directly responsible for the above effects is unknown. However, this elevated E-Asp-AT activity in OC users is evidence of a possible redistribution of PAL-P within the body and may reflect an increased requirement of vitamin B_6 by this group of women.

GENERAL DISCUSSION

A classical method in the determination of the requirement for a particular nutrient is establishment of a basal or normal level of that nutrient or some parameter that reflects its status, depletion of body stores of the nutrient, and then repletion with known levels until the basal level is once again reached. This was the general procedure followed in the present study.

Results from E-PAL analysis showed that only after an intake of vitamin B_6 at the 5.06 mg/day level were original values restored to normal in all 8 subjects. In contrast to the present study, the work of Fu (43) indicated that E-PAL levels in nonusers of OC were completely restored to initial levels by a daily intake of 1.62 mg of vitamin B_6. Comparison of the response of both users and nonusers of OC showed that nonusers had a much greater increase in E-PAL per milligram of vitamin B_6 consumed, after having had their body stores depleted.

Analysis of E-Ala-AT activity and in vitro percent stimulation also reflected an increased need for vitamin B_6 by the OC users of the present study. Deprivation of dietary vitamin B_6 for 32 days resulted in a significant decline in mean E-Ala-AT activity which was restored to 94% of the mean initial value by an intake of vitamin B_6 at the 5.06 mg level. Results from percent stimulation with in vitro PAL-P indicated again that vitamin B_6 intake at the 5.06 mg level had not fully restored initial values in all subjects.

Enzyme activity of E-Asp-AT and percent stimulation with in vitro PAL-P in these same subjects did not respond to either depletion of or repletion with supplemental amounts of vitamin B_6. The reliability of this parameter for assessment of vitamin B_6 nutriture in women using OC

is questioned.

An intake of 2.0 mg of vitamin B_6 per day is recommended by RDA (USA) (74) for adults consuming 100 g of protein or more. The vitamin B_6 requirement for young adult women, based on urinary excretion and erythrocyte levels of the vitamin, and E-Asp-AT activity and stimulation with in vitro PAL-P in a study by Fu (43) was determined as 1.5 mg/day. The present study, based on parameters reflecting vitamin B_6 status in the erythrocyte, indicates that at least some OC users require vitamin B_6 in amounts that are greater than levels recommended for either adults in general or for young adult women. Although no precise amount can be stated, it is proposed that the requirement is between 1.5 and 5 mg of vitamin B_6 per day. In view of the estimate that many North American diets supply less than 1.0 mg of vitamin B_6 per day (75) and the findings of Driskell et al. (76) that 3/4 of the female students in a study to determine vitamin B_6 requirements consumed less than the 2.0 mg/day recommended by RDA (USA) (74), an amount of 5 mg of vitamin B_6 could be met only by some form of supplementation; perhaps in pill form or by fortification of foods.

The observation that OC users might require higher intakes of vitamin B_6 to restore E-PAL and E-Ala-AT to initial levels following depletion of body stores is an indication that OC usage in some way alters vitamin B_6 metabolism. The estrogen progesterone combination might directly alter vitamin B_6 metabolism in the erythrocyte, but it is more likely that the erythrocyte reflects altered metabolism of the vitamin in the entire system or perhaps in a specific organ. Several mechanisms for the action of OC on vitamin B_6 metabolism have been proposed, but conclusive evidence as to the exact nature of this effect

remains to be ellucidated. Among the possible explanations are:

A. Effect of estrogens on the adrenal cortex:

Although the use of OC had not yet become widespread, Sandberg and Slaunwhite (77) in 1959 provided evidence that the estrogenic component of these preparations was capable of altering cortisol metabolism by increasing the binding capacity of corticosteroid - binding - globulin (CBG) for glucocorticoids, thus increasing blood levels of these protein bound glucocorticoids. Keller et al. (78) confirmed the above finding and further demonstrated that although these bound glucocorticoids were inert, they could enter hepatic cells by pinocytosis and dissociate from the CBG to yield active, free glucocorticoids. Supporting evidence comes from recent studies reporting increases in plasma and serum levels of total (bound and unbound) glucocorticoids during therapy with combined estrogen-progesterone preparations (79, 80). There is now evidence that in addition to bound glucocorticoids, levels of free glucocorticoids are increased during OC therapy (81, 82). This modifying influence of estrogens upon adrenal function might have several ensuing effects on enzyme systems:

1) Tryptophan oxygenase (TO), the first and rate limiting enzyme in the tryptophan to nicotinic acid pathway is glucocorticoid inducible. Braidman and Rose (38) have shown that estrogens stimulate TO in rat liver. Altman and Greengard (83) demonstrated that administration of hydrocortisone in humans caused marked increases in hepatic TO levels, as determined by tissue biopsy, and was accompanied by increased urinary excretion of kynurenine. In view of the evidence supporting the hypothesis of estrogen-mediated increases in bound and unbound glucocorticoids, it is possible that estrogens can influence TO activity indirectly by their

effect upon glucocorticoid levels. The net effect would be an accelerated turn-over rate of the tryptophan to nicotinic acid pathway with an increase in the requirement for PAL-P - the coenzyme necessary for several interconversions in the pathway.

Rose and McGinty (84) studied cortisol induction of TO in human subjects and found that cortisol administration produced elevated excretion of the following tryptophan metabolites: XA, KYN, 3-OH-KYN, and 3-OH-ANTH after a load dose of 2 g of tryptophan. Urinary excretion of tryptophan metabolites following a similar load dose of tryptophan in users and nonusers of OC, determined in the present study but not reported here, showed that the OC users excreted significantly higher levels of XA, KYN and 3-OH-KYN. Analysis for 3-OH-ANTH was not done. The increased requirement for vitamin B_6 in this study, determined by E-PAL and E-Ala-AT activity, thus could have been a reflection of a functional vitamin B_6 deficiency induced by the increased capacity for the conversion of tryptophan to nicotinic acid and thus a subsequent greater need for the coenzyme. 2) Braidman and Rose (38) have shown that estrogens produce increases in 2 other corticoid - inducible enzymes: tyrosine aminotransferase (Tyr-AT) and Ala-AT in rat liver. Although it is not known whether OC usage produces similar Ala-AT induction in human liver, Rose and Cramp (39) have demonstrated a decrease in plasma tyrosine concentration in OC users, an effect attributable to increased catabolism of this amino acid by Tyr-AT. Aly et al. (16) found significantly lower levels of total plasma amino acids in OC users when compared to nonusers. The decrease was due principally to markedly decreased non-essential plasma amino acids. Other researchers (40, 85) have indicated diminished levels of total plasma amino acids in OC users. Holt (86) found no significant

elevation in total urinary nitrogen in OC users, thus, the decreased plasma amino acid levels appear to be due, not to increased catabolism, but perhaps to increased tissue utilization.

It is possible that OC agents increase other PAL-P dependent amino-acid metabolizing pathways indirectly through an estrogen-mediated increase in corticoid activity in the liver, thus causing a redistribution of tissue vitamin B_6 to the liver. Lefauconnier et al. (87) have shown that repeated injection of cortisol into female rats did increase liver Tyr-AT activity. Beare-Rogers et al. (88) demonstrated that estrogen - containing OC administration to female rats produced elevated Ala-AT activity in the liver. In both studies, no increase in actual liver content of vitamin B_6 was shown. The increased activities in these PAL-P dependent enzymes, however, could indicate a greater need for the coenzyme. If such a mechanism occurs in the human, it could possibly account for the need for larger amounts of vitamin B_6 to restore E-PAL and E-Ala-AT in the OC users of the present study after having had their body stores depleted.

B. Direct effects of estrogens on enzymes:

Estrogens can affect enzymes which are not mediated by way of adreno-cortical hormones. Brin (89) examined adrenalectomized - ovariectomized female rats and found reduced TO and Ala-AT activity. Only TO was re-stored by estrogen administration, indicating that TO was influenced directly by estrogens, while the effect on Ala-AT was secondary to an adrenocortical response. Braidman and Rose (38) demonstrated that adrenalectomy reduced TO and Tyr-AT activity in female rat liver, and that estrogen treatment only partially restored the activity of these enzymes

to control values. They suggested that adrenocortical steroids were required for full expression of estrogen effects on TO and Tyr-AT, but that estrogens also exerted action on these enzymes which was not mediated by way of the adrenocortical steroids. Thus, it is possible that other enzymes, in addition to those inducible by glucocorticoids, may be affected by OC usage. This explanation could possibly account for the significantly elevated E-Asp-AT activity shown by OC users.

C. Enzyme inhibition by estrogen conjugates:

Mason et al. (90) have demonstrated that sulfate esters of estrogens interfere both in vitro and in vivo with the activity of the PAL-P dependent enzymes - kynureninase and kynurenine aminotransferase, by competing with the coenzyme for sites on the apoenzyme molecule. Large doses of PIN-HCl reversed this inhibition by a "mass action" effect. This interference of estrogen conjugates has been cited as one of the mechanisms for altered tryptophan metabolism characteristic of many OC users (91), as it does explain the increased 3-OH-KYN and XA excretion following a tryptophan load dose. This mechanism, however, would not be expected to affect tissue levels of vitamin B_6.

The increased requirement for vitamin B_6 by OC users, as reflected by E-PAL and E-Ala-AT determinations is most likely a combination of apoenzyme induction, coenzyme redistribution within tissues, and possibly enzyme inhibition. Two important observations, however, must be kept in mind. The first is that although OC users required higher levels of vitamin B_6 to restore E-PAL and E-Ala-AT to initial levels after depletion of their body stores, their pre-depletion values were not significantly

different from those found in nonusers of OC. Analysis of E-Asp-AT in the 2 groups confirmed the previous finding that OC users have significantly elevated E-Asp-AT levels; an observation which does not reflect vitamin B_6 deficiency. The second factor to be considered is that the abnormalities in urinary tryptophan metabolites occur only after the pathway is stressed with a load dose of tryptophan. While on a daily vitamin B_6 intake of 2.06 mg and no tryptophan load, there was no evidence of a subclinical deficiency of vitamin B_6 in the OC users of the present study. However, when an element of stress, like a dietary deficiency of the vitamin or amino acid loading was imposed on the OC users of this study, abnormalities characteristic of vitamin B_6 deficiency did appear. That these abnormalities were corrected by doses of vitamin B_6 greater than the 2.0 mg recommended as adequate for the normal adult woman (11) is an indication of an increased requirement for the vitamin.

Possible implications of an increased vitamin B_6 requirement during OC treatment:

A. Depression has been frequently reported as one of the side effects of OC use (37, 92-94) and in some cases has been alleviated by vitamin B_6 supplementation (37, 92, 95). A frequently proposed hypothesis to explain the mechanism of OC induced depression is that of decreased 5-hydroxytryptamine (5-HT) synthesis from tryptophan in the brain. This decreased synthesis is thought to arise from:

1) the increased TO activity which pours tryptophan down the nicotinic acid pathway and away from the 5-HT pathway, and

2) the functional deficiency of vitamin B_6 created by the increased TO activity, leaving inadequate amounts of the coenzyme necessary for normal 5-hydroxytryptophan decarboxylase activity in the brain. Since depression

is often a cause for discontinuation of OC usage, the therapeutic value of vitamin B_6 warrants further investigation.

B. The tryptophan load test has revealed abnormal tryptophan metabolism in pregnancy that was correctable by the administration of vitamin B_6 (96). Plasma levels of PAL-P (97-99) and E-Asp-AT activity (99, 100) have also been reported to be lower in pregnant women than in controls and have been restored to normal levels by vitamin B_6 administration. The rising levels of endogenous estrogens during pregnancy are believed to increase tryptophan metabolism mediated through a similar glucocorticoid induction as described in OC users (100). An additional mechanism has been proposed by Brin (101). Analysis of maternal and cord blood for vitamin B_6 content and aminotransferase activity showed higher levels in cord blood, suggesting that the fetus concentrates vitamin B_6. A woman who had been previously ingesting OC could enter pregnancy with already depleted stores of vitamin B_6 and would thus be less able to meet the increased demands for the vitamin during pregnancy. As a result, the fetus as well as the mother could be at risk as animal studies have shown lower body weights, slower physical development and impaired neuromotor development (102); as well as gross physical abnormalities (103) in rat pups born to female rats maintained on vitamin B_6 deficient diets. The vitamin B_6 status should be considered at the start of pregnancy, particularly in subjects who have previously used oral contraceptives.

Results from the present study support the findings of others (17, 104) that the need for vitamin B_6 by OC users is increased. Based on normalization of E-PAL concentration and E-Ala-AT activity after depletion of body stores of vitamin B_6, the amount of the vitamin necessary

was between 1.5 and 5 mg per day. This amount is lower than the 20 to 30 mg per day proposed by several workers (17, 18, 35, 36) to normalize tryptophan metabolism, but could be greater than the 2.0 mg recommended for normal women (72) and greater than the calculated average daily vitamin B_6 intake of 1.62 mg by young adult women (43).

SUMMARY

1. The pre-study clinical analyses showed all subjects to be in good health. Hemoglobin levels in the experimental and control groups were normal and were not significiantly different from each other. All subjects showed normal S-Asp-AT levels and none showed evidence of excess urinary glucose.

2. Hemoglobin and hematocrit levels in both experimental and control groups were not affected by the amount of vitamin B_6 consumed.

3. A comparison of E-PAL concentration in experimental and control subjects after 1 day on the adjustment diet, containing 2.06 mg of vitamin B_6 per day, showed a significantly higher level in the experimental group which could have been due to a higher intake of vitamin B_6 previous to the study. After 10 days on the adjustment diet, both groups stabilized at very similar E-PAL concentrations. Thus, no decrease in E-PAL concentration could be attributed to OC use while subjects were consuming approximately 2 mg of vitamin B_6.

4. During depletion of the experimental subjects, an initial insignificant, upward shift in mean E-PAL concentration was followed by a steady decline to a value at the end of depletion which was 49% of the mean undepleted value. In repletion phase 1, a daily intake of 0.96 mg of vitamin B_6 for 7 days increased mean E-PAL to 74% of the mean initial value. After a daily intake of 1.56 mg of the vitamin for 8 days in repletion phase 2, mean E-PAL rose to 104% of the mean initial value. This level of repletion had restored initial E-PAL levels in 5 of the 8 subjects. An intake of 5.06 mg of vitamin B_6 for 6 days in repletion phase 3 succeeded in restoring E-PAL concentrations to original levels in all subjects.

5. E-Ala-AT analyses showed the experimental group as having slight, but

insignificantly higher mean levels of enzyme activity than the control group both after 1 and 10 days of consuming the adjustment diet. Mean values for percent stimulation of E-Ala-AT with in vitro PAL-P, both after 1 and 10 days on the adjustment diet, were lower in the experimental group when compared to the control group.

6. After 32 days of consuming the low vitamin B_6 diet, the experimental group showed a significant decline in mean E-Ala-AT activity to a value which was 45% of the mean undepleted activity. Phase 1 of repletion produced only a slight increase in E-Ala-AT to 47% of the mean undepleted activity. Phase 2 and 3 of repletion succeeded in increasing mean enzyme activity to 77% and 94% of the mean undepleted activity, respectively. Although mean percent stimulation of E-Ala-AT with in vitro PAL-P fluctuated somewhat, the general trend was one of increasing percent stimulation as depletion progressed and decreasing percent stimulation with repletion of vitamin B_6.

7. Mean E-Asp-AT activity after 1 day on the adjustment diet was similar in both the experimental and control group. After 10 days on the same diet, the experimental group showed a significantly elevated mean E-Asp-AT activity when compared to the control group. No significant differences were detected in percent stimulation of E-Asp-AT with in vitro PAL-P between the 2 groups either after 1 or 10 days on the adjustment diet.

8. Basal activity and percent stimulation of E-Asp-AT of the experimental subjects, as determined in this study did not consistently reflect the intake of vitamin B_6, either during depletion or supplementation with vitamin B_6. The reliability of this parameter in assessing vitamin B_6 nutritional status in women using OC was questioned.

9. The results of the E-PAL and E-Ala-AT determinations in this study indicated that OC users, while consuming approximately 2 mg of vitamin B_6 per day did not differ markedly from nonusers. Thus, utilizing the above parameters; no evidence of a spontaneous vitamin B_6 deficiency in this group of OC users was detected. Once, however, their body stores of the vitamin were depleted, a higher level of supplemented vitamin B_6 was required to restore E-PAL concentration and E-Ala-AT activity to pre-depletion levels than is recommended as adequate for the normal adult woman. The amount required was between 1.5 and 5 mg of vitamin B_6 per day.

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APPENDIX I

The Low Vitamin B_6 Diet

Basal Diet

	g/day
Breakfast	
Knox flavored orange gelatin	20
Omelet	
Egg white, raw	70
Cheddar cheese, grated	10
Bread, white	69
Jelly, apple	30
Lunch	
Soup	
Tomato soup, condensed	100
Gelatin, dry	7.5
Sandwich	
Bread, white	46
Cheese, Canadian, processed	28
Salad	
Grapefruit sections, canned	60
Mandarin orange sections, canned	20
Lettuce, fresh	10
Apple, fresh	50
Dinner	
Casserole	
Noodles, egg, processed[a]	100
Beef, ground chuck, processed[b]	35
Tomato soup, condensed	60
Cheese, Canadian, processed	28
Green beans, processed[c]	60
Jellied salad	
Pears, canned	30
Peaches, canned	30
Jello, dry	28
Cream, whipping	30

a 270 gm of dry noodles were boiled for 10 minutes in 5 quarts of hot tap water and drained. This procedure was repeated 3 times.

b 1000 gm of thawed ground beef was divided into approximately equal portions and placed into each of 2 large kettles. Eight quarts of warm tap water were added to each kettle and the meat was broken up into small pieces, boiled for 15 minutes, and drained. The 2 batches of meat were mixed together and autoclaved at 18 lb pressure for 20 minutes. Portions were frozen until ready for use.

c Six quarts of warm tap water were added to 3 15½ oz tins drained French style green beans. The beans were boiled for 20 minutes and drained.

Ad Libitum Diet

	Weight of each piece g
Candies	
Mints	8.6
Hard Candies	5.7
Butter crunch	10.0
Shortbread cookies[a]	10.0
Sugar	
Butter	
Gingerale[b]	
Coffee[c]	
Tea[d]	

a The calculated vitamin B_6 content for each 10 g of raw dough was 3 µg.

b Gingerale was introduced on the 16th day of the study.

c Both instant and regular perked coffee were allowed.

d Only instant tea was allowed.

N.B. Coffee and/or tea were allowed up to a total intake of 4 cups per day.

APPENDIX II

Microhematocrit

1. Fill each microhematocrit tube 2/3 full with blood. If blood has not been recently drawn, mix well by inversion so that a representative sample can be taken. Do in duplicate. Place tubes in rack in appropriate section for each subject.
2. Plug end, which has not been wet with blood, with a small amount of modeling clay.
3. Place in order in slots in microcentrifuge head. Make sure that the plugged end is against the outer circumference of the head.
4. Centrifuge for 5 minutes.
5. Remove each tube and measure percent packed cells using the microhematocrit reader.
6. Average 2 readings.

APPENDIX III

Separation of Plasma and Packed Red Blood Cells (RBC)

N.B. After collection, keep blood samples in ice to prevent deterioration.

1. Label round bottom tubes (16 x 100 mm), that have been previously marked at the 2 ml level, with subject code. Divide each 10 ml sample of whole blood into 4-2 ml portions. i.e. 4 tubes for each subject.
2. Centrifuge at 600 rpm/10 minutes in a Sorvall SS3 centrifuge. This is to pack the RBC but leave the white blood cells (WBC) suspended in the supernatant plasma.
3. Using a glass hook attached to a rubber mouth piece, remove the plasma and WBC from the packed RBC. Composite for each subject in a 28 x 100 mm plastic centrifuge tube and retain for amino acid analyses.
4. To each tube of packed RBC add 2.0 ml 0.9% saline to wash the cells. Mix gently by inversion, covering mouth of tube with parafilm.
5. Centrifuge at 1,200 rpm for 5 minutes in Sorvall SS3.
6. Gently remove the supernatant wash water with a glass hook and discard.
7. Use tubes 1 and 2 for Ala-AT and Asp-AT analyses. Calculate volume of packed RBC in 2 ml blood, using % packed cells determined by the micro-hematocrit method. Add glass distilled water in ratio of 4 parts water to 1 part of packed cells. Gently mix to hemolyze cells and transfer contents to uncalibrated tubes of the same size. Cap with parafilm covered cork and label. Freeze.
8. Use tubes 3 and 4 for E-PAL analyses. Bring volume to 2 ml with saline (0.9%). Label and cap as in (7). Freeze, protecting from light.

APPENDIX IV

Microbiological Assay of Erythrocyte Pyridoxal Concentration

A. MATERIALS

1. Assay Organism: Saccharomyces carlsbergensis 4228; ATCC No 9080.

2. Reviving Culture: Aseptically add to the freeze-dried culture 0.3 to 0.4 ml of Saboraud's Dextrose broth with a Pasteur pipette; mix well and transfer the total mixture to a test tube containing 5-6 ml of the same broth. Transfer the last few drops to an agar slant. Incubate at 25° - 30° C for several days, or until luxuriant growth is obtained.

3. Stock Culture: Once the new culture is growing, transfer it to and maintain on slants of Difco malt agar. Incubate for 24 hours and store in the refrigerator. Transfer the stock culture onto fresh slants at least once every two weeks.

4. Stock Solutions for Basal Medium

a) Acid hydrolyzed casein: Vitamin-free acid hydrolyzed casein was purchased from Nutritional Biochemicals, Inc., and was found to be very satisfactory. Sterilize each time after use.

b) Potassium citrate - citric acid buffer solution: Dissolve 125 g potassium citrate ($K_3C_6H_5O_7-H_2O$) and 62.5 g citric acid ($H_3C_6O_7-H_2O$) in distilled water. Bring volume to 1 liter.

c) Salt solution I: Dissolve in distilled water

21.0 g KCl

6.3 g $CaCl_2-2H_2O$

6.3 g $MgSO_4-7H_2O$

0.13 g $FeCl_3$-$6H_2O$

0.13 g $MnSO_4$-H_2O

Add 0.5 ml concentrated HCl and dilute with distilled water to 1 liter. To hasten solution, stir with a magnetic stirrer.

d) Salt Solution II: Dissolve in distilled water

50 g $(NH_4)_2$ HPO_4

28 g KH_2PO_4

Add 0.5 ml of concentrated HCl and dilute with distilled water to 1 liter.

e) Vitamin Solution I: Dissolve in 0.02 N acetic acid:

13.0 mg thiamine-HCl

1.3 g inositol

Add 0.02 N acetic acid to make 1 liter. Store in refrigerator.

f) Biotin Stock Solution: Dissolve 10 mg biotin in 100 ml of 50% ethanol: water. This solution contains 100 µg biotin/ml. Store in refrigerator.

g) Vitamin Solution II: Dissolve in distilled water

125 mg calcium pantothenate

125 mg nicotinic acid

Add 5 ml of biotin stock solution and make to 1 liter with distilled water. Store in refrigerator.

5. Basal Medium: For 250 ml of basal medium add the items listed in the following table to approximately 100 ml of distilled water.

Solution	Amount
Acid hydrolyzed casein	20 ml

Potassium citrate buffer	20 ml
Vitamin solution I	10 ml
Vitamin solution II	10 ml
Salt solution I	10 ml
Salt solution II	10 ml
DL-Tryptophan[a]	38 mg
Anhydrous dextrose[b]	25 g

Heat solution gently to help to dissolve the dextrose. Adjust pH to 4.0-4.5 with NaOH or HCl and dilute to 250 ml with distilled water. Shortly before the medium is to be used, sterilize by steaming for 10 minutes. Prepare the medium daily, but if needed it may be refrigerated for 1 or 2 days. The medium as prepared is "double strength." Therefore, dilute it 1:1 by adding water, sample, or standards to the tubes.

6. Standard Solutions[c]

a) Stock solutions: 500 µg/ml

Form of Vitamin B_6	Weight[d]
pyridoxine-HCl	121.6 mg
pyridoxal-HCl	121.8 mg
pyridoxamine-2HCl	143.4 mg

Dissolve the above amounts separately in 25% ethanol and make up to 200 ml with additional 25% ethanol. Store in dark bottles at about 10° C.

b) Dilution I: 10 µg/ml

Dilute 5 ml pyridoxal stock solution to 250 ml with 25% ethanol. Store

a Add tryptophan to about 25 ml of distilled water, heat gently to help to dissolve, and add to mixture.

b Bacto-dextrose from Difco Company was satisfactory.

c Prepare all solutions containing any free form of vitamin B_6 under a red light.

d B_6 vitamins should be previously dried to constant weight in the dark in a desicator.

in dark bottle and renew monthly.

c) Dilution II: 100 ng /ml

Dilute 2 ml of dilution I to 200 ml with 25% ethanol. Store in dark bottle and renew weekly.

d) Working standard: 2 ng /ml

Dilute 2 ml of dilution II to 200 ml with distilled water. Prepare the working standard fresh for each assay.

7. Culture and Suspension Media

a) Agar slants: Dissolve 9 g Difco malt agar in 200 ml distilled water by steaming. Dispense 7-8 ml per tube and stopper. Sterilize by autoclaving for 15 minutes at 15 lb pressure. Slant and cool. Store in the refrigerator.

b) Inoculum Broth: To 100 ml of basal medium add an equal volume of distilled water. Dispense 10 ml per tube, stopper, sterilize by autoclaving for 15 minutes at 15 lb pressure. Cool and store in the refrigerator.

8. Preparation of Inoculum: Transfer aseptically a loopful of yeast from the stock culture into a tube containing 10 ml of the inoculum broth. Incubate 6 hours at 30° C. To minimize the possibility of contamination, use a slant only once, then discard. Therefore, make a transfer from the stock culture to a new slant each time the assay is run.

After incubation, transfer culture to a sterile centrifuge tube and spin in a Sorval SS-3 centrifuge at 2000-1500 rpm for 10 minutes. Decant supernatant, and suspend cells in 10 ml of sterile suspension medium. Repeat this procedure until cells have been washed twice with suspension medium, then resuspend in a third 10 ml portion. This is the inoculum.

9. Preparation of the Samples: While protecting from light, thaw the tubes containing the erythrocyte hemolysate.

Transfer thawed hemolysate to 50 ml erlenmeyer flasks, using a minimal amount of distilled water to rinse tubes. To each flask add 12 ml 10% TCA and swirl. Let flasks stand for 30 minutes, swirling them occasionally. Transfer the contents of each flask to centrifuge tubes and spin at room temperature at 2500 rpm for 30 minutes. Decant supernatant into clean 50 ml erlenmeyer flasks and autoclave at 15 lb pressure for 30 minutes.

Cool, adjust pH to 5.2 with dilute KOH and bring volume to 25 ml with distilled water.

The samples can be prepared a day in advance and stored in the dark in the refrigerator.

10. Preparation of Reagents

a) 3N HCl: Add 189 ml of 12N HCl to approximately 500 ml distilled water. Make up to 1 liter with distilled water.

b) Stock potassium acetate (1 M): Dissolve 49.7 g potassium acetate in distilled water to make 500 ml.

c) 0.01 M potassium acetate: Prepare fresh for each assay. Add 2.5 ml of the stock solution to 200 ml of distilled water, adjust pH to 4.5 with acetic acid and bring the final volume to 250 ml.

d) 0.02 M potassium acetate: Prepare fresh for each assay. Add 5 ml of the stock solution to 200 ml distilled water, adjust pH to 5.5 with acetic acid, and bring the final volume to 250 ml.

e) 0.04 M potassium acetate: Prepare fresh for each assay. Add 10 ml of the stock solution to 200 ml distilled water, adjust pH to 6.0 with acetic acid and bring the final volume to 250 ml.

f) 6N KOH: Dissolve 396 g KOH (85%) in 1000 ml distilled water. Work under a fume hood and add KOH slowly to water to avoid splattering.

11. Preparation of the resin

Use Dowex 50 AG WX 8 (100 - 200 mesh) in the H^+ form. Weigh out 400 g dry resin and suspend in distilled water to make a thick slurry. Add excess 6N KOH until the pH of the supernatant is above 8. Check this measurement with pH paper. Let the resin settle and decant the KOH. Rinse the resin with water until the supernatant liquid is colorless. Add 400 ml of 3N HCl and heat the resin for 30 minutes with constant stirring in a boiling water bath. An automatic stirrer facilitates this. Remove the resin from the heat, allow to settle and decant the supernatant. Repeat the 3N HCl treatment twice more. Rinse the resin with distilled water until the pH of the rinse water is 4 to 5. Add excess 6N KOH (about 400 ml) until the supernatant liquid is well above pH 8. Rinse resin with distilled water until supernatant liquid is pH 9 (pH meter) The final rinse water should be decanted and the resin left in 0.02 M potassium acetate until used.

The used resin is collected and regenerated, beginning with 3N HCl step.

12. Preparation of the columns:

Rinse a sufficient amount of resin (6 ml per column) with distilled water to remove the potassium acetate. This takes about 6 liters of water. The final rinse water should be pH 7. Suspend resin in distilled water. Support the column on a ring stand and tap a plug of glass wool into the bottom of the column. Add a small amount of distilled water and tap again to remove any air bubles. Measure a 6 ml portion of the settled resin into each of the columns. Allow resin to settle and gently insert a small plug of glass wool above the resin. Drain the water in the column to the top of the upper glass wool plug.

Do not allow the level of the liquid to fall below this point.

Rinse each column with 4-10 ml portions of hot (75° C) distilled water, and twice with 10 ml portions of hot (75° C) 0.01M potassium acetate. The pH of the potassium acetate eluant should be 4.5. If not, rinse with a third portion. The column is now ready for application of the sample or standard which should be applied while the column is still warm.

B. CHROMATOGRAPHY[a]

1. Preparation of standard:

Dilute 10 ml of the 100 ng /ml stock solution of pyridoxal (see Appendix II.A. 6.d) with about 70 ml distilled water. Adjust pH to 5.2 with dilute KOH and bring volume to 100 ml with distilled water.

2. Chromatography of samples:

Using a 4 ml volumetric pipette apply each sample in duplicate to each of two columns. Allow the sample to drain completely into the warm column with unrestricted flow. As soon as the level of the liquid has just reached the upper plug of glass wool, wash the reservoir and sides of the column with 2-10 ml portions of hot (75° C) 0.02M potassium acetate. This eluant is discarded. Pyridoxal is eluted with 2-10 ml portions of boiling 0.04 M potassium acetate.

Collect elute in a 25 ml volumetric flash, adjust pH to 4.5 - 5.0 using dilute acetic acid, and bring volume to 25 ml with distilled water.

3. Chromatography of Standard PAL:

Because of the slight variation in slope of the standard curve

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a The entire chromatographic separation is carried out under red illumination.

from day to day, a standard curve is run with each assay.

Pipette 5 ml of the PAL standard (see Appendix IV B. 1) on to a prepared column and elute the pyridoxal fraction according to the procedure outline above. Collect the pyridoxal fraction in a 25 ml volumetric flash. Adjust pH to 4.5 - 5.0 with dilute acetic acid and bring volume to 25 ml with distilled water. Use pH paper to test the pH. The pyridoxal eluate theoretically contains a total of 50 ng or 2 ng pyridoxal per ml.

C. ASSAY PROCEDURES[a]

1. Preparation of the tubes:

The tubes for the assay are pyrex culture tubes (25 x 100mm) which have been matched in a Bausch and Lomb Spectronic 20. Number the tubes and arrange in neoprene coated racks which fit a constant temperature water bath shaker (see Appendix IV. C. 3). Add sample or standard, water, medium and inoculum to the tubes as outlined on page 108.

When sample or standard and water have been added to all tubes, add a glass bead to each tube and cap with a plastic cover that fits loosely. Steam tubes and basal medium, which is in a separate container, for 10 minutes. Cool to room temperature and aseptically add 5 ml basal medium to each tube using a 5 ml syringe.

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a The assay must be done under red light.

Number of tubes	Concentration PAL, ng/tube	Eluate ml/tube	H_2O ml/tube	Basal Medium, ml	Inoculum 1 drop
PAL Standard					
1	----	----	5.0	5	X
2	----	----	5.0	5	✓
2	0.2	0.1	4.9	5	✓
2	0.6	0.3	4.7	5	✓
2	1.0	0.5	4.5	5	✓
2	2.0	1.0	4.0	5	✓
2	3.0	1.5	3.5	5	✓
2	4.0	2.0	3.0	5	✓
Sample					
2		0.5	4.5	5	✓
2		1.0	4.0	5	✓
2		2.0	3.0	5	✓

2. Inoculation:

Aseptically inoculate all but 1 blank tube with 1 drop of freshly prepared inoculum into each tube (see Appendix IV. A. 8). Use a sterile insulin syringe fitted with a number 24 needle. Inoculate no more than 10 tubes at one time before refilling syringe.

3. Incubation:

Incubate both inoculated tubes and uninoculated blanks at 25° - 30° C for 22 hours with constant shaking, using a Precision Scientific shaking water bath (serial number 13-v-2). Use a stroke rate of 100/ minute. Cover the top of the water bath to protect the tubes from light.

4. Reading the tubes:

After incubation immediately read the percent transmittance (% T) of each tube in a Bausch and Lomb Spectronic 20 at 550 mμ. If reading cannot be done immediately, the tubes may be steamed for 5 minutes to arrest growth.

Zero the instrument with the uninoculated blank. The inoculated blanks should have about 85% T compared with the uninoculated blank. Adjust the instrument to 100% T with the inoculated blanks and read all other tubes against the blank. Immediately before reading, each tube must be mixed for 5 seconds on a Vortex Jr. mixer at speed number 4. Allow bubbles to rise before reading. After reading, autoclave all tubes for 15 minutes at 20 pounds pressure before discarding contents.

5. Calculations:

Average the duplicate % T readings of the tubes containing PAL standard and plot against concentration of PAL (ng) using semi-logarithmic paper. Determine by interpolation the amount of PAL in the sample tubes, calculate the amount of PAL on the basis of 1 ml of packed red blood cells and report values as an average of the six tubes.

ng PAL/ml packed RBC=

$$\frac{\text{ng PAL/tube x erythrocyte hemolysate dilution factor}}{\text{\% Packed RBC}} \times 100$$

APPENDIX V

Determination of Erythrocyte Alanine Aminotransferase Activity and Percent In Vitro Stimulation with PAL-P.

A. REAGENTS

1. Alanine Substrate:

1.78 g dl-alanine

2.0 g potassium monophosphate (KH_2PO_4)

0.6 g -ketoglutarate

Dissolve in glass distilled water and adjust pH to 7.4 with dilute KOH, using a pH meter. Bring final volume to 100 ml.

2. Potassium monophosphate buffer (0.1 M):

Dissolve 1.36 g KH_2PO_4 in glass distilled water, and adjust pH to 7.4 using a pH meter. Bring final volume to 100 ml with glass distilled water.

3. 100% Trichloroacetic Acid (TCA):

Dissolve 100 g of TCA in 100 ml glass distilled water (Weigh the TCA quickly as it is very hydroscopic).

4. Dinitrophenylhydrazine reagent (DNPH):

Dissolve 100 mg 2,4-DNPH in a mixture of 20 ml concentrated HCl and 80 ml glass distilled water.

5. Alcoholic KOH:

Dissolve 2.5 g KOH in 100 ml 95% ethanol.

6. Stock solution of pyridoxal phosphate (500 µg/ml):

Dissolve 26.82 mg PAL-P-$2H_2O$ in glass distilled water and bring volume to 50 ml. Pipette 2.5 ml portions of this stock solution into small brown storage bottles, seal tightly, and store in the dark at -20 C. This amount is sufficient for stimulation of 24 tubes.

7. Standard sodium pyruvate solution (1mg/ml):

Dissolve 100 mg Na-pyruvate in glass distilled water and bring to volume of 100 ml.

B. STANDARD NA-PYRUVATE CURVE

1. Na-pyruvate working standards:

Dilute with glass distilled water the specified amounts of stock Na-pyruvate as outline below:

Stock Standard (ml)	Dilute to (ml)	Concentration of Na-pyruvate µg/ml	µg/tube
1.0	50	20	10
3.0	50	60	30
4.0	50	80	40
3.0	25	120	60
4.0	25	160	80

Pipette 0.5 ml of each diluted standard into duplicate tubes to give the concentration listed above (µg/tube).

2. Proceed with the determination as outlined in the Procedure on page 112, adding the reagents to the tubes in the sequence listed.

3. Read optical density of the toluene-alcoholic KOH mixture at 490 mµ in a Beckman DU-2 spectrophotometer.

4. For the standard curve, average the duplicate optical density values obtained and plot against corresponding Na-pyruvate concentration.

C. DETERMINATION OF E-ALA-AT ACTIVITY IN RBC HEMOLYSATES.

1. Preparation of erythrocyte hemolysate:

Thaw hemolyspate containing 1 part cells to 4 parts water (see Appendix III.7). Centrifuge in a Sorvall SS-3 for 15 minutes at 2500 rpm to remove debris.

Procedure for determination of basal and stimulated E-Ala-AT activity.

		Hemolysate		
	Standard	Blank	Sample	In vitro stimulation
1) Add				
Buffer	0.5 ml	0.5 ml	0.5 ml	0.4 ml
PAL-P				0.1 ml
Hemolysate		0.5 ml	0.5 ml	0.5 ml
Na-pyruvate	0.5 ml			
100% TCA		1 drop		
Substrate	0.5 ml	0.5 ml	0.5 ml	0.5 ml
2) Mix by gentle shaking				
3) Incubate		37° C for 60 minutes		
4) Add				
100% TCA	1 drop		1 drop	1 drop
DNPH reagent	1.0 ml	1.0 ml	1.0 ml	1.0 ml
5) Mix with Vortex				
6) Let stand exactly 5 minutes				
7) Add toluene	1.0 ml	1.0 ml	1.0 ml	1.0 ml
8) Mix with Vortex for 10 seconds				
9) Centrifuge for 5 minutes				
10) Transfer 0.5 ml aliquots of toluene layer to clean tubes				
11) Add 2.5% alcoholic KOH	2.0 ml	2.0 ml	2.0 ml	2.0 ml
12) Mix				
13) After 15 to 20 minutes, transfer to absorption cells and read optical density at 490 mμ in a Beckman DU-2 spectrophotometer.				

Into each of 8 tubes pipette 0.5 ml of the hemolysate; 3 tubes for enzyme activity, 3 for PAL-P stimulation and 2 for blanks.

2. Addition of reagents: Working with 8 tubes at one time add reagents to the tubes in the sequence listed in the Procedure on page 112.

3. Transfer toluene-alcoholic KOH misture to absorption cells and read optical density at 490 mμ in a Beckman DU-2 spectrophotometer.

4. Calculations: The amount of pyruvate formed in each tube was determined through use of a standard curve. E-Ala-AT activity is expressed as μg pyruvate/ml RBC/hr.

Calculation of percent stimulation:

$$\% \text{ Stimulation} = \frac{\mu\text{g pyruvate/ml RBC/hr with in vitro PAL-P} - \mu\text{g pyruvate/ml RBC/hr}}{\mu\text{g pyruvate/ml RBC/hr}} \times 100$$

APPENDIX VI

Description of the Study Conducted by Fu (43) on Nonusers of Oral Contraceptives.

Subjects: Eight healthy female graduate students ranging in age from 21 to 30 years, varying in height from 5 ft. to 5 ft. 8 in. and in weight from 114 to 138 lb were studied.

Diet: The diet used by Fu was identical to the one used in the present study (see Appendix I) except that Fu's diet contained 46 g less bread and 20 g less apple jelly. Fu's diet was analysed to contain 0.34 mg of vitamin B_6/day. In both studies all food was processed, cooked and served in the same way.

Timing of the Study:

Days on Study	Phase	Vitamin B_6 in Diet[a]	PIN-HCl Supplement[a]	Total B_6 Intake[a]	Days Intake Consumed
43	Depletion	0.34	-	0.34	43
50	Repletion I	0.34	0.6	0.94	7
53	Repletion II	0.34	1.2	1.54	3
54	Load Dose	0.34	30	30.34	1
64	Repletion III	Ad libitum	-	X = 1.62	11
				range = 1.37 - 1.94	

a Expressed in mg.

Blood Samples: Fasting blood samples of approximately 10 ml each were taken by venipuncture from the arm of each subject on days 2, 9, 16, 23, 30, 37, 44, 51 and 54 of the study and the 10th day post-study. The samples were prepared for analysis in the same manner as described in the present study (see Appendix III).

Methods of Analysis:

Erythrocyte vitamin B_6: A protozoological method (Baker, H., O. Frank, M. Ning, R. Gellene, S. M. Hunter and C. M. Leevy. A protozoological method for detecting clinical vitamin B_6 deficiency. Am. J. Clin. Nutr. 18: 123, 1966) was used for determination of vitamin B_6 in red blood cell hemolysates.

Aminotransferase activity and PAL-P stimulation: Both E-Ala-AT and E-Asp-AT were determined spectrophotometrically by the kit method (from C. F. Boehringer and Soehne Gmbh, Manheim, Germany. Instructions on measurement of GOT and GPT activities are included). The PAL-P stimulation of both aminotransferases was done according to the method suggested by Cheney et al. (51).